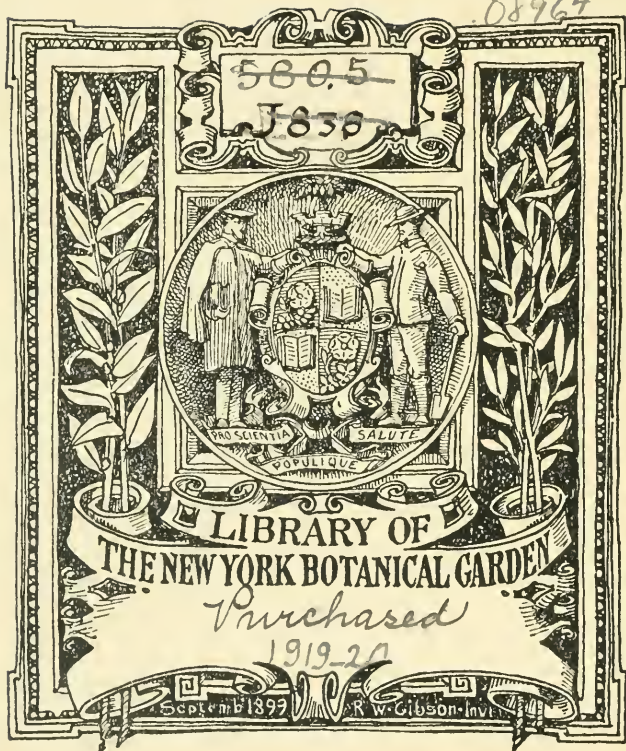


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THE JOURNAL OF
GENERAL PHYSIOLOGY

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

VOLUME SECOND

WITH 1 PLATE AND 224 FIGURES IN THE TEXT



NEW YORK

THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

1920

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v. 2
1919-20

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WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

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THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

VOLUME II, NO. 1

SEPTEMBER 20, 1919



PUBLISHED BIMONTHLY

AT 2419-21 GREENMOUNT AVE., BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. Information regarding contributions and subscriptions is given in full on the back cover.

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COMPARATIVE STUDIES ON RESPIRATION.

VII. RESPIRATION AND ANTAGONISM.

INTRODUCTORY NOTE.

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, June 15, 1919.)

The relation of antagonism to such fundamental life processes as respiration and photosynthesis has received scant attention. The writer began studies on this subject some years ago, but owing to other interests the investigations did not progress beyond the preliminary stage. It seemed desirable that they should be carried forward, and this was facilitated by the development of new methods of measuring respiration and photosynthesis.¹

A series of studies has recently been commenced in which these methods have been employed. Care was taken to make frequent measurements, as earlier work had shown this to be important. The fact that the rate of respiration could be determined as often as once every 3 minutes made it possible to obtain satisfactory time curves.

The plan of these investigations involves a comparative study of the antagonistic effects of salts on different kinds of organisms under precisely the same conditions. In this way it is hoped that a sound basis for the formulation of general principles may be provided.

It may be of interest to mention certain improvements in technique. When a reagent is employed which has a buffer effect it is desirable to have the same buffer action during the measurement of normal respiration as during exposure to the reagent. This may be accom-

¹ Haas, A. R. C., *Science*, 1916, xliv, 105. Osterhout, W. J. V., and Haas, A. R. C., *J. Gen. Physiol.*, 1918-19, i, 1. Osterhout, W. J. V., *J. Gen. Physiol.*, 1918-19, i, 17, 171.

plished by introducing the reagent into the apparatus² in a separate tube, having an outlet which is flush with the inside of the stopper. When the time comes to bring the reagent into contact with the organism the tube is inverted and the current of air carries the reagent over into the tube containing the organism. In order that the height of the liquid in the latter tube may not be unduly raised it may be provided at the bottom with rubber tubing, into which, after thorough mixing, some of the liquid may be received and clamped off.

If the reagent is volatile it is necessary to substitute for it (during the measurement of normal respiration) a non-volatile liquid having the same buffer action.³ The volatile reagent may be placed in the rubber tubing, attached to the tube containing the organisms, and clamped off until the time comes to bring it into contact with the organisms. The clamp is then opened and, after thorough mixing, the height of the liquid is adjusted as described above.

Since respiration is affected by acidity and alkalinity it is desirable to add an indicator to the liquid containing the organisms in order to follow its reaction. The color of this indicator may be affected by the color of the organism or by that of the medium, but in most cases it affords useful information regarding changes in the reaction of the liquid.

There have been few previous investigations on this subject. Warburg⁴ found that the consumption of oxygen by fertilized eggs of the sea urchin (*Strongylocentrotus*) fell off when the eggs were transferred from sea water to a solution of NaCl (isotonic with sea water). This is explained by the fact, previously discovered by Loeb,⁵ that NaCl causes cytolysis of the eggs. Loeb had found that this injury could be prevented by the addition of a trace of NaCN. Warburg accordingly added NaCN to the solution of NaCl and found that the amount of oxygen consumed was five times the normal. Addition of CaCl₂ brought it back to the normal amount.

² For a description of this see Osterhout, W. J. V., *J. Gen. Physiol.*, 1918-19, i, 17.

³ See Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxv, 237.

⁴ Warburg, O., *Z. physiol. Chem.*, 1910, lxxvi, 305; *Biochem. Z.*, 1910, xxix, 414.

⁵ Loeb, J., *Die chemische Entwicklungserregung des tierischen Eies*, Berlin, 1909.

Loeb and Wasteneys⁶ pointed out that Warburg's interpretation of his results is of doubtful value, owing to complications introduced by the addition of NaCN. In experiments on the fertilized egg of *Arbacia* Loeb and Wasteneys⁶ found that there was no increase in the consumption of oxygen in a solution of NaCl (without NaCN) even when due allowance was made for the percentage of eggs killed by the action of the solution. In NaCl + KCl the eggs consumed approximately the normal amount of oxygen⁷ and the addition of Ca was practically without effect.

Meyerhof⁸ repeated Warburg's experiments on *Strongylocentrotus*, using a solution of NaCl (without NaCN) and making determinations after 12, 15, or 20 minutes, during which short periods little injury occurs. He found that the consumption of oxygen was from two to four times as great as in sea water. The addition of CaCl₂ reduced the consumption of oxygen to the normal. The addition of KCl to NaCl had practically no effect.

Similar experiments have apparently not been performed upon plants.

The investigations thus far completed in the writer's laboratory show a much closer agreement with the results of Loeb and Wasteneys in regard to NaCl than with those of Warburg and Meyerhof. They also show pronounced antagonism between such substances as NaCl and CaCl₂ in their effect on respiration.

Reports of some of these investigations will appear in the near future.

⁶ Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1910, xxviii, 340; 1911, xxxi, 168.

⁷ The experiment lasted an hour, during which time the solution has little injurious action.

⁸ Meyerhof, O., *Biochem. Z.*, 1911, xxxiii, 291.

COMPARATIVE STUDIES ON RESPIRATION.

VIII. THE RESPIRATION OF *BACILLUS SUBTILIS* IN RELATION TO ANTAGONISM.

By MATILDA MOLDENHAUER BROOKS.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, June 15, 1919.)

Although the antagonistic effects of salts on certain bacteria have been studied, no attempt has been made to ascertain whether any relation exists between these effects and the respiration. The investigations described in this paper were undertaken with a view to obtaining some light on this question.

The first attempt to study antagonism in relation to bacteria was made by Lipman.¹ He used the production of NH_3 by *Bacillus subtilis* as an index of metabolism. He allowed cultures of *Bacillus subtilis* to grow over night in various salts and combinations of salts in the proportions found in sea water, and measured the production of NH_3 . There was a considerable decrease in the production of NH_3 when the salts were used singly, but this did not occur when combinations of salts were made in the proportions found in sea water.

Winslow and Falk² have observed antagonistic effects in experiments on *Bacillus coli*. These investigators found that cultures suspended in solutions of NaCl or CaCl_2 were decreased in number; that higher concentrations produced sterilization of the culture; and that a combination of NaCl and CaCl_2 in the molecular proportions of 5 : 1 was favorable to the growth of the organism.

¹ Lipman, C. B., *Bot. Gaz.*, 1909, xlviii, 105; 1911, xlix, 41.

² Winslow, C.-E. A., and Falk, I. S., *Proc. Soc. Exp. Biol. and Med.*, 1918, xv, 67.

Shearer³ has also demonstrated similar effects of salts upon the viability of meningococcus and *Bacillus coli*. He found that a combination of NaCl and CaCl₂ was favorable to growth, while each salt used separately produced decrease in growth.

It is evident, therefore, that antagonistic effects are to be expected in studying bacteria, and it seemed desirable to investigate these effects in relation to respiration. For this purpose the apparatus described by Osterhout⁴ was employed.

The organism selected was *Bacillus subtilis*, the same strain as that used in an investigation previously reported.⁵ It had originally been isolated from water and grown daily upon agar-agar. In every case an 18 hour culture, inoculated upon agar-agar with a few cc. of 0.75 per cent dextrose solution, was used. These inoculations were incubated at 37°C. and the resulting heavy growth of bacteria was washed off with 0.75 per cent dextrose solution and centrifugated to get rid of any foreign substances that may have surrounded the bacteria. The bacteria were then suspended in 0.75 per cent dextrose solution and were ready for experimentation. It is very important to use cultures not older than 24 hours, as the respiration of the older cultures is considerably diminished.

In making up the solution of dextrose and the salts, distilled water was employed. The salts used were NaCl, CaCl₂, and KCl in molecular concentrations from 0.05 M to 1.0 M. Experiments on MgCl₂, which belong to this series, are in process of completion.

The temperature varied in the course of the experiments from 18–20°C.

The indicator used was 5 drops of 0.01 per cent phenolsulfonephthalein in 10 cc. of water. Tap water was used, as distilled water has a pH value not exceeding 7.1 and this was not alkaline enough for measuring with this indicator. Buffer solutions were made from boric acid and borax, having pH values of 7.78 and 7.60 respectively. These buffers were used as the standard for comparison in determining the color change produced in the indicator as the CO₂ was driven over.

³ Shearer, C., *Proc. Roy. Soc. London, Series B*, 1917, lxxxix, 440; *Proc. Camb. Phil. Soc.*, 1919, xix, 263.

⁴ Osterhout, W. J. V., *J. Gen. Physiol.*, 1918–19, i, 17, 171.

⁵ Brooks, M. M., *J. Gen. Physiol.*, 1918–19, i, 193.

For experimentation, 2 cc. of the emulsion of bacteria were placed in the apparatus, and the air was caused to circulate. The CO_2 produced by the bacteria was thereby carried over into the indicator, and the reciprocal of the time required to change the color from pH 7.78 to 7.60 was taken as the rate of respiration or production of CO_2 . When the indicator had reached the end-point required, the stop-cock was closed, thus allowing the CO_2 to be washed out of the system again by passing the air through the NaOH , and thereby returning the indicator to its original color. In this way, a series of readings could be taken. In general these were remarkably constant. It was found that the rate of respiration, under normal conditions (*i.e.* of bacteria placed in 0.75 per cent dextrose solution), was practically constant for about 6 hours. The experiments, however, lasted only 70 minutes. This included the time necessary for the establishment of the normal rate, usually 10 minutes, and the time for determining the effect of the salt.

When the normal rate had been determined, the salt was added and the change in the rate was observed. The first reading was discarded owing to the possibility of experimental error, as the result of CO_2 dissolved in the salt solution. In adding the salt solution, the system was opened because it was found that no appreciable error was introduced by exposing the system for a moment to contact with the air.

When the bacteria had been in contact with the salt for an hour, the respiration seemed to have reached an equilibrium, as the rate then decreased very slowly during the next few hours. This decrease was faster in the higher concentrations of the salt; the lower concentrations remained constant for hours at a time. In adding the salt, 2 cc. (of double the strength desired for experimentation) were added to 2 cc. of the bacteria in 0.75 per cent dextrose solution, so that the volume remained constant throughout all the experiments.

It is generally known that a reduced pressure of oxygen has little effect for a considerable time upon the rate of oxidation, so that the slight change in the oxygen content of the system during the experiment does not introduce an experimental error.

Fig. 1 shows the manner in which the rate of CO_2 production changes under the influence of NaCl in the concentrations of 0.15,

0.1, 0.5, 0.8, and 1.0 M. During the first 10 minutes the bacteria are under normal conditions and the curve (broken line) is horizontal. After this (at the point marked 0 on the abscissa) the salt is added.

Rate of CO_2 production

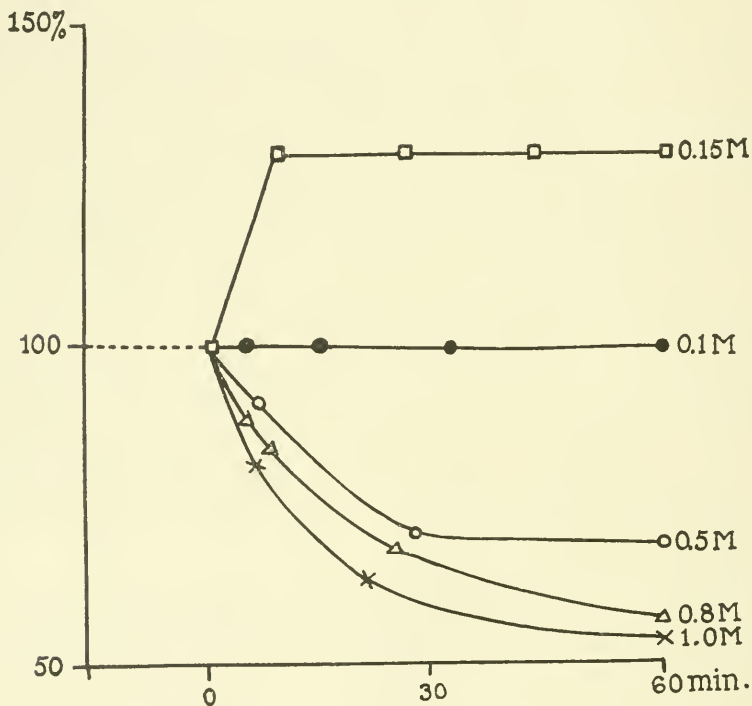


FIG. 1. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.15, 0.1, 0.5, 0.8, and 1.0 M NaCl. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

For example, the addition of sufficient NaCl to make the concentration 0.15 M produces a rise in the rate, which remains constant during the period of experimentation. When the concentration of NaCl

is 0.1 M the rate is normal, while in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

Rate of CO_2 production

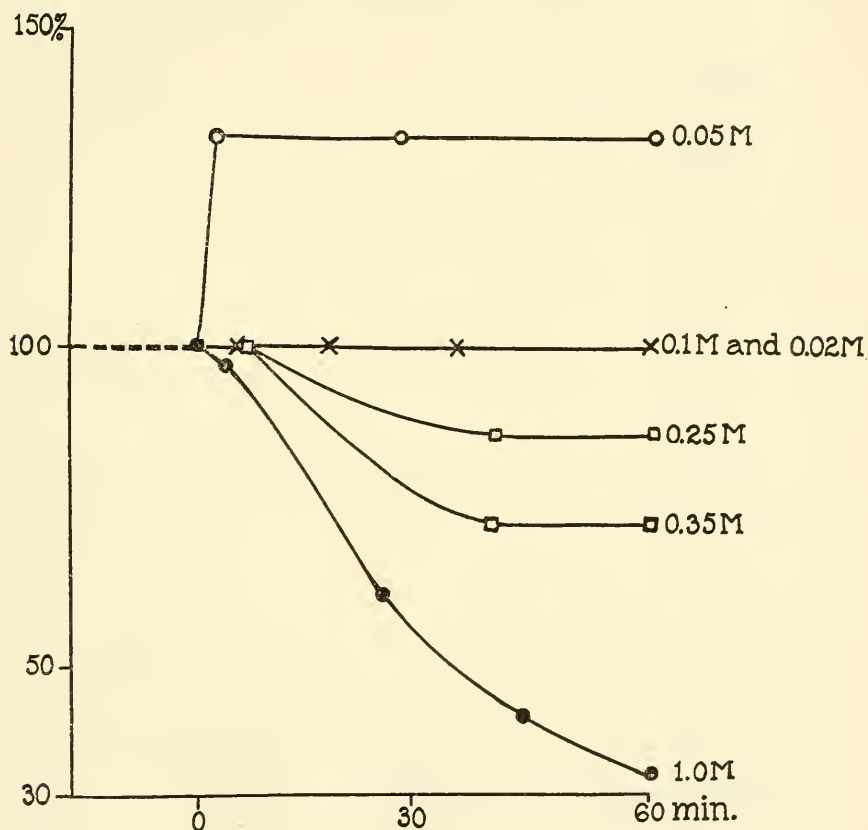


FIG. 2. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.05, 0.1, 0.25, 0.35, and 1.0 M CaCl_2 . The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

Fig. 2 shows the manner in which the rate of CO_2 production changes under the influence of CaCl_2 in the concentrations of 0.02, 0.05, 0.1, 0.25, 0.35, and 1.0 M. In 0.01, 0.02, and 0.1 M the rate is normal; in 0.05 M there is an increase in rate; in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

Rate of CO_2 production

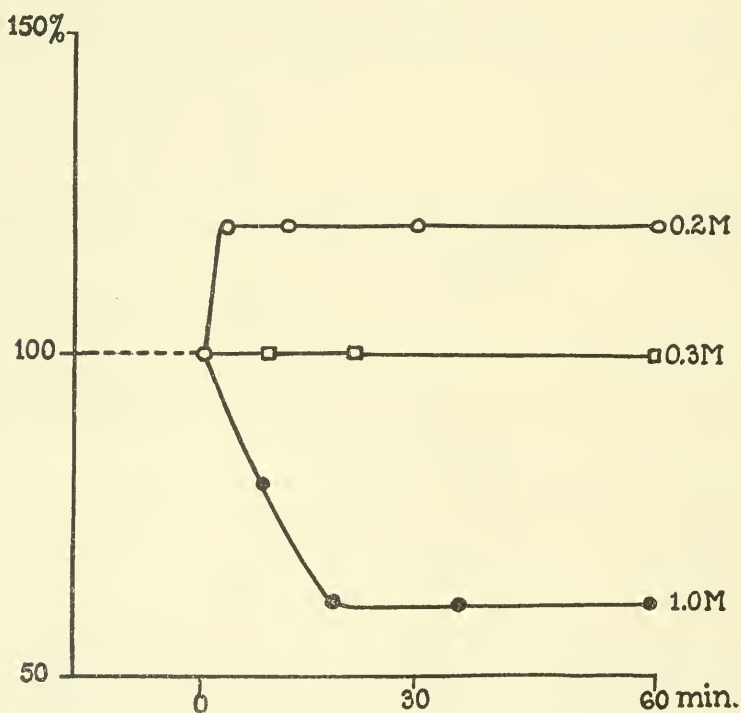


FIG. 3. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.2 M, 0.3 M, and 1.0 M KCl. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension, usually 30 seconds. Each curve represents a single typical experiment.

Fig. 3 shows the manner in which the rate of CO_2 production changes under the influence of KCl in concentrations of 0.2, 0.3, and 1.0 M. When the concentration is 0.2 M, there is an increase in the rate which remains constant for some time; in lower concentrations (0.15 and 0.1 M) the rate is normal, while in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves, and each represents one experiment.

Fig. 4 shows the effects of various concentrations of NaCl, KCl, and CaCl_2 upon the rate of respiration expressed as per cent of the normal rate. The rate indicated is that produced after the bacteria had been in contact with the salt for 1 hour. The figure shows that NaCl produces an increase in the rate of respiration at a concentration of 0.15 M. In 0.5, 0.8, and 1.0 M there is a decrease. KCl produces an increase in the rate at a concentration of 0.2 M, and in concentrations higher than 0.3 M it causes a decrease. CaCl_2 causes an increase in the rate at a concentration of 0.05 M and in concentrations higher than 0.1 M it causes a decrease in respiration. CaCl_2 is the most toxic of the salts used, while KCl is the least toxic; this agrees with the results of Lipman.¹ It is of interest to note that there is evidently a correlation between the production of NH_3 of the organism (as found by Lipman) and the rate of production of CO_2 as shown here.

Fig. 5 shows the antagonism of salts. Thus, Curve A shows that when five parts of NaCl and one part of CaCl_2 (in the same molecular concentrations) were added to the bacteria, the rate of respiration remained normal, or as if no salt had been added. This was true only when the proportions of 5:1 were used. When other proportions were used the respiration decreased accordingly, and gave only a fraction of the normal rate. This agrees with the results of Winslow and Falk² on the growth of *Bacillus coli*, but not with those of Lipman,¹ on the production of NH_3 by *Bacillus subtilis*, who found no antagonism between NaCl and CaCl_2 .

Curve B illustrates the effect of combinations of KCl and NaCl upon the rate of respiration. There are two maxima in this curve; one at 4 KCl to 6 NaCl, and the other at 6 KCl to 4 NaCl. The former is the more nearly normal, although there is no combination

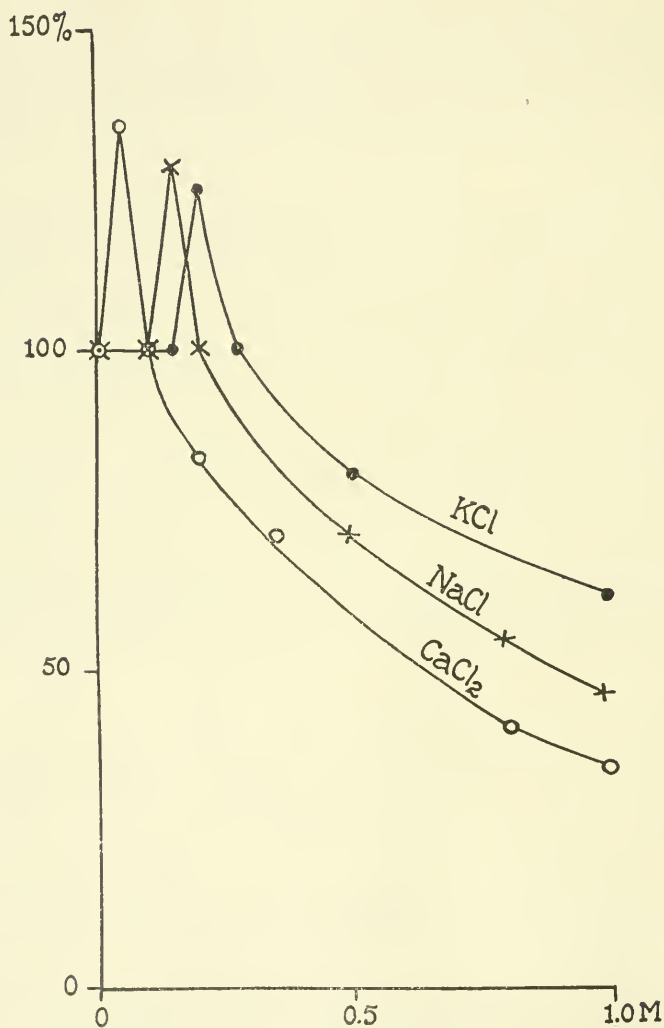
Rate of CO_2 production

FIG. 4. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) as effected by salts. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Average of three experiments; probable error less than 3 per cent of the mean.

of these two salts (in the concentrations used) that produces normal respiration. It is of interest to note that Osterhout⁶ and Lipman¹ also obtained two maxima with these salts in experiments upon wheat

Rate of CO₂ production

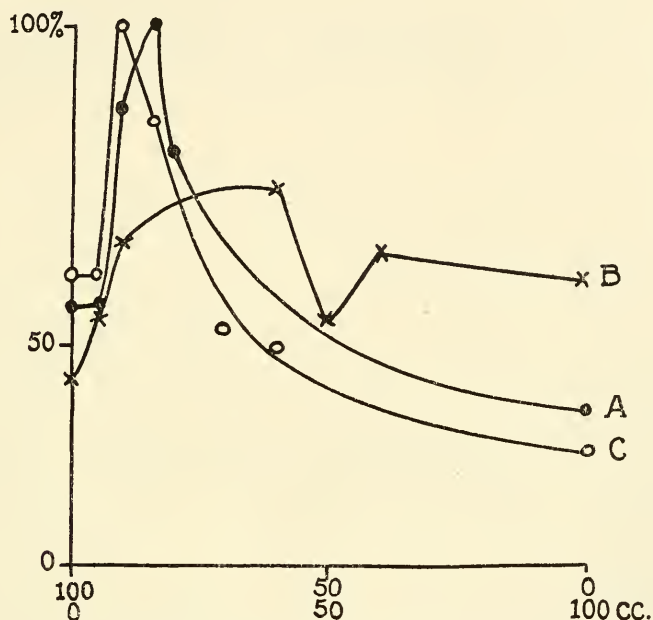


FIG. 5. Curves showing antagonism in the effect of salts on the respiration of *Bacillus subtilis*. Curve A, antagonism between NaCl, 0.8 M (left), and CaCl₂, 0.8 M (right); Curve B, antagonism between NaCl, 1 M (left), and KCl 1 M (right); Curve C, antagonism between KCl, 1 M (left), and CaCl₂, 1 M (right). The ordinates represent rate of respiration (expressed as per cent of the normal); the abscissæ represent molecular proportions of the salts used. Thus, in Curve A, the ordinate at the extreme left represents the rate in NaCl 0.8 M, while the ordinate at the extreme right represents the rate in CaCl₂ 0.8 M. The ordinate in the middle represents the rate in 50 parts NaCl 0.8 M + 50 parts CaCl₂ 0.8 M. The normal rate (which is taken as 100 per cent) represents a change in pH from 7.78 to 7.60 in about 30 seconds, varying according to the number of bacteria used. Curve A, average of two experiments; Curve B, average of five experiments; Curve C, average of three experiments. Probable error less than 3 per cent of the mean.

⁶ Osterhout, W. J. V., *Bot. Gaz.*, 1909, xlviii, 98.

and upon the production of NH_3 by *Bacillus subtilis* respectively. The fact that both salts are monovalent may be a factor in explaining their peculiar behavior.

Curve C of Fig. 5 shows antagonism between KCl and CaCl_2 . This was also observed by Lipman¹ in the production of NH_3 . The maximum effect is found at 9 KCl to 1 CaCl_2 , where the rate of respiration is 100 per cent. In comparing this curve with Curve A (NaCl and CaCl_2) one can readily observe that more KCl is required to antagonize CaCl_2 than would be required of NaCl. It is interesting to observe that KCl is the least toxic of the three salts, and that it is the least effective in influencing respiration. This agrees with the results of Lipman¹ on the production of NH_3 by *Bacillus subtilis*.

There are no similar investigations on the respiration of plants with which a comparison might be made. Some interesting studies have been made on sea urchin eggs by Warburg,⁷ by Loeb and Wasteneys,⁸ and by Meyerhof,⁹ an account of which is given in a recent summary by Osterhout.¹⁰ The results obtained with bacteria agree with those of Loeb and Wasteneys in that there is no rise in rate in NaCl, except in 0.15 M concentration in which the rise is only 30 per cent which is insignificant compared with that obtained by Warburg⁷ and Meyerhof⁹ (200 to 500 per cent). On the whole the results are more nearly in agreement with those of Loeb and Wasteneys.⁸

In order to find out what effect was produced on the bacteria while they were being acted upon by the salts, a few recovery experiments were tried. After the bacteria had remained in the salt solution for an hour, they were centrifugated and thoroughly washed in dextrose solution and centrifugated again. The supernatant fluid was then drained off, 2 cc. of dextrose solution were added, and their respiration was measured. It was found that within a period of less than $\frac{1}{2}$ hour the rate became normal.

A control experiment was made by substituting the same salt in the same molecular concentration as was removed from the bacteria

⁷ Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305; *Biochem. Z.*, 1910, xxix, 414.

⁸ Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1910, xxviii, 340; 1911, xxxi, 168.

⁹ Meyerhof, O., *Biochem. Z.*, 1911, xxxiii, 291.

¹⁰ Osterhout, W. J. V., *J. Gen. Physiol.*, 1919-20, ii, 1.

after centrifugating them to see whether or not the mechanical manipulation was responsible for the normal rate. The rate, however, showed no recovery under these conditions.

In order to find out whether the pH value of the liquid containing the bacteria changed when the salts were added, thereby influencing the rate of respiration, an indicator was added to this liquid in the apparatus and the pH value was observed to remain so nearly constant that the change in the rate of respiration could not be attributed to changes in alkalinity of the medium in which the bacteria were placed.

SUMMARY.

1. In relatively low concentrations of NaCl, KCl, and CaCl₂ the rate of respiration of *Bacillus subtilis* remains fairly constant for a period of several hours, while in the higher concentrations, there is a gradual decrease in the rate.

2. NaCl and KCl increase the rate of respiration of *Bacillus subtilis* somewhat at concentrations of 0.15 M and 0.2 M respectively; in sufficiently high concentrations they decrease the rate. CaCl₂ increases the rate of respiration of *Bacillus subtilis* at a concentration of 0.05 M and decreases the rate at somewhat higher concentrations.

3. The effects of salts upon respiration show a well marked antagonism between NaCl and CaCl₂, and between KCl and CaCl₂. The antagonism between NaCl and KCl is slight and the antagonism curve shows two maxima.

COMPARATIVE STUDIES ON RESPIRATION.

IX. THE EFFECTS OF ANTAGONISTIC SALTS ON THE RESPIRATION OF *ASPERGILLUS NIGER*.

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(Received for publication, July 21, 1919.)

The relation of antagonistic salts to the respiration of higher fungi has received no attention. As the problems involved are of considerable interest the writer has made a beginning in this direction by conducting a series of experiments on *Aspergillus niger*.

It may be of interest to compare the results with those obtained on other organisms.

In experiments on sea urchin eggs Warburg¹ and Meyerhof¹ found that NaCl causes a rise in respiration which is inhibited by the addition of CaCl₂. Loeb and Wasteneys¹ found no such rise. The results with *Aspergillus* show a rise with lower concentrations of NaCl and a fall with higher concentrations. Since the highest rise in NaCl (24 per cent) is very small as compared with the rise of several hundred per cent obtained by Warburg and by Meyerhof, the results as a whole agree much more nearly with those of Loeb and Wasteneys. Still closer agreement is found in the results of Brooks,² who has used both sodium and calcium in studies on the respiration of bacteria. She finds an increase in the rate of respiration with certain concentrations of NaCl and CaCl₂ and a decrease in higher concentrations. A mixture of NaCl and CaCl₂ shows antagonism.

In some experiments on *Aspergillus niger* Kosinski³ tested the effect of NaCl; although he seems to think that a 1.523 per cent (0.26M)

¹ For a summary of the experiments on sea urchin eggs see Osterhout, W. J. V., *J. Gen. Physiol.*, 1919-20, ii, 1.

² Brooks, M. M., *J. Gen. Physiol.*, 1919-20, ii, 5.

³ Kosinski, I., *Jahrb. wiss. Bot.*, 1902, xxxvii, 154.

solution does not affect respiration, his data show an increase in respiration of 41 per cent.

The writer, in his experiments on *Aspergillus niger*, used NaCl and CaCl₂, as well as mixtures of the two salts. For measuring the production of CO₂ the apparatus described by Osterhout⁴ was employed, in which the material is placed in one tube and the indicator in another.⁵ This method is very accurate and simple. Care must be exercised to prevent any alkali from passing over from the tube containing it into the indicator tube, as this would vitiate the results.

By adding an indicator to the tube containing the fungus it can be shown that an acid is produced which is non-volatile (or practically so) since it does not disappear when a current of air (free from CO₂) is passed through the liquid for 15 or 20 minutes (under these conditions CO₂ would disappear in 5 minutes or less).

The apparatus is so constructed that a non-volatile acid cannot affect the color of the indicator which is being matched by the observer. The question may arise, however, whether the production of such an acid has any bearing on the interpretation of the results. If it is borne in mind that the problem is to ascertain the changes produced in the output of CO₂ under the influence of reagents it is evident that we need not consider the production of other acids except as intermediate stages or as by-products whose study is a problem by itself. This problem always exists, for wherever respiration goes on organic acids and other substances are produced. It seems best for the present to confine the investigation to the production of CO₂, leaving the study of other substances to the future.

The rate of production of CO₂ is obtained by taking the reciprocal of the time required to produce a definite change of color in the indicator tube.⁶

The fungus was grown and handled as previously described,⁶ except that water distilled from a hard glass flask was used in place of tap water. The fungus, which forms a mat on the surface of the cul-

⁴ Osterhout, W. J. V., *J. Gen. Physiol.*, 1918-19, i, 17, 171.

⁵ In this investigation five drops of a 0.01 per cent solution of phenolsulfonphthalein were added to 10 cc. of water. This solution was then used for comparison with the buffer tubes, which contained the same amount of indicator.

⁶ Gustafson, F. G., *J. Gen. Physiol.*, 1918-19, i, 181.

tured solution, was rinsed in distilled water⁷ to free it from any adhering nutrient solution, before putting it in the apparatus. The proper amount of material was then wound around the glass tube dipping into the solution in the respiration tube and secured in this position by tying it with a thread. This exposed the surface of the fungus to the liquid and also kept it from moving about and insured the smallest amount of mechanical injury. When the apparatus was in motion the air bubbles passing through the solution containing the organism carried the CO₂ given off by the fungus into the indicator tube, where the change in color was noted.

In all experiments pH 7.75 was the starting point and pH 7.42 the end-point. This gave the same range for every reading and a uniform change of 0.33 pH. The time required to produce this change under normal conditions varied from $2\frac{1}{4}$ to $3\frac{1}{2}$ minutes, depending upon the amount and condition of the material.

A number of preliminary experiments were performed without giving the fungus any nutrient while they lasted. When no nutrient was present the rate of respiration gradually fell below normal. For this reason it was thought best to add enough food to keep the control up to normal, so that any deviation from normal would be due to the action of the salt under investigation.

In starting an experiment the rate of respiration was first obtained in a 0.1 per cent solution of dextrose in distilled water. Unless the rate remained practically constant for at least 20 minutes in this solution, the material was rejected. The rate obtained in the dextrose solution is called the normal rate of respiration and in all calculations is taken as 100 per cent.

Several concentrations of dextrose were tried, but as all seemed to give the same results 0.1 per cent was chosen, as low viscosity is advantageous in the prevention of foaming. When the reagent used in the experiment was introduced the concentration of dextrose was thereby diluted one-half, so that during the experiment with the reagent it was only 0.05 per cent. In the numerous controls which were made the same thing was done, but no effect on the respiration was

⁷ All water used in these experiments was distilled from a hard glass flask, and came in contact with hard glass only throughout the experiment.

noticed, and control experiments made in this way kept up to normal for several hours, or as long as the experiment lasted.

Various concentrations of NaCl were used. Lower concentrations such as 0.125M, 0.25M, and 0.5M caused a rise in respiration. At 1M the results were rather variable, some experiments showing a rise and others a fall. Solutions of 2M always gave a decided decrease which was followed by a small increase. The respiration then remained constant for more than an hour or to the end of the experiment. Figs. 1 and 2 give a graphic representation of the results obtained with various concentrations of NaCl.

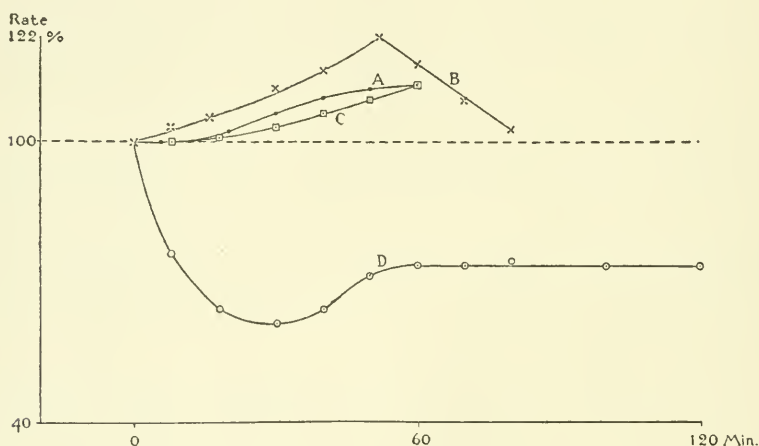


FIG. 1. Respiration of *Aspergillus niger*. The broken straight line to the left of the point marked 0 on the abscissa represents the normal rate of respiration before the addition of the salt. Curve A represents the respiration in 0.125M NaCl, Curve B respiration in 0.25M NaCl, Curve C respiration in 0.5M NaCl, Curve D respiration in 2M NaCl, and the broken line the control in 0.05 per cent dextrose. A, B, and C were in 0.05 per cent dextrose while D was in 1.5 per cent dextrose. Curves A, C, and D are each an average of 3, B of 4 experiments. Probable error less than 3 per cent of the mean.

The results with the lower concentrations are somewhat variable as to the amount of increase, as well as in respect to the time of maximum respiration. Thus in some experiments with 0.25M NaCl the maximum was reached at about 30 minutes after the introduction of the salt, while in most the maximum was not reached until at the end of 50 minutes.

This variability was of course to be expected with substances that are not more toxic than NaCl, where the least individual variation in the physiological activity of the cultures has a chance to exert its influence fully. This fact has already been noted in respect to some of the weaker anesthetics.⁶ Though there are differences in the amount of increase and in the time when the maxima occur, yet there

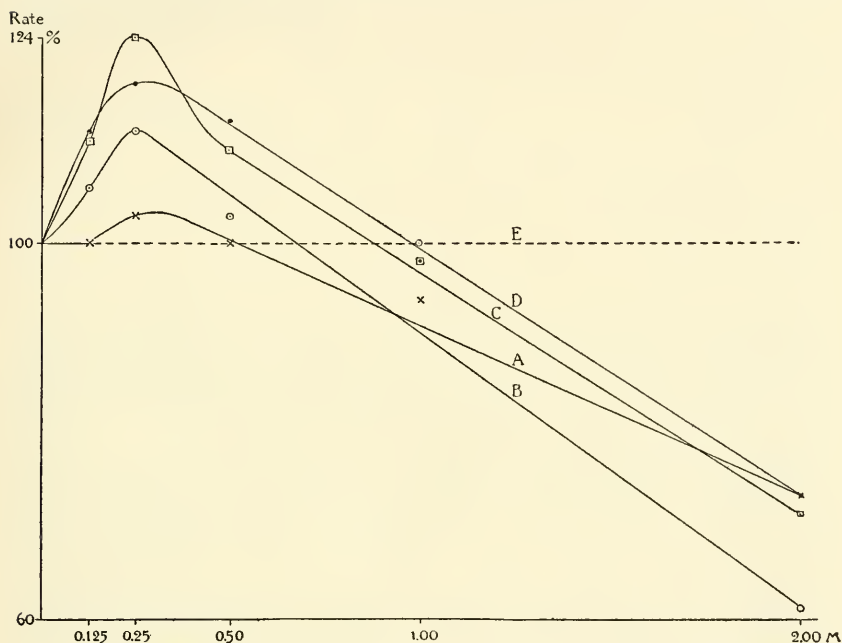


FIG. 2. Respiration of *Aspergillus niger*. The curves show rate plotted against concentration and represent the same experiments as shown in Fig. 1. Curve A represents the respiration at the end of 10 minutes exposure to NaCl, Curve B at end of 30, Curve C at end of 52, and Curve D at end of 60 minutes. Curve E represents the control in 0.05 per cent dextrose.

is no doubt about the general result. There is a distinct, though small, amount of increase in the rate of respiration, when *Aspergillus niger* is treated with NaCl at concentrations between 0.125 and 0.5M.

With 1M solutions of NaCl apparently contradictory results were obtained; *i.e.*, some experiments gave a large initial decrease followed by a rise to normal, others gave little or no initial decrease followed

by a rise above normal, while still other experiments gave only normal respiration.

The writer believes that these results are due to two factors acting on the fungus. One is the specific chemical action of NaCl which tends to stimulate, while the other, the osmotic pressure of the NaCl, tends to decrease the respiration. As is well known the abstraction of water from tissues lowers the rate of respiration. The fact that sometimes one factor prevails, and sometimes the other is probably due to physiological differences in the fungus.

Experiments with 2M NaCl show a large initial decrease followed by an increase of about 10 per cent. This is also found with 1M but is not so pronounced. The explanation of this fact may be that at first rapid withdrawal of water occurs, but as the salt penetrates and osmotic pressure within the cell increases, water is taken up, causing a rise in respiration. This supposition is further strengthened by experiments with 1.25M CaCl_2 ,⁸ which showed a large initial decrease, not followed by an increase in respiration, but by a slow steady decrease which might be expected if CaCl_2 does not penetrate readily. Osterhout⁹ in his experiments on *Laminaria* showed that NaCl increases the permeability, while CaCl_2 at first decreases it.

Only one concentration of CaCl_2 was used with dextrose, but several more concentrations without dextrose were employed. The concentration used with dextrose was 0.5M. This caused an increase in the respiration, giving a curve with rounded apex as shown in Fig. 3, Curve B. Concentrations used without dextrose were 0.3125M, 0.625M, and 1.25M. The first two caused a rise, while the last one only a decrease in respiration.

At this point it may be of interest to note that the effect of the salt seemed to be more pronounced when dextrose was absent than when it was present. This was especially the case with CaCl_2 . Kosinski found that *Aspergillus niger* does not store up raw food material, and that as soon as it is taken out of a solution containing nutrient material it is in a starving condition.¹⁰ He states that when this happens

⁸ This concentration of CaCl_2 has approximately the same osmotic pressure as 2M NaCl.

⁹ Osterhout, W. J. V., *Science*, 1911, xxxiv, 187; 1912, xxxv, 112.

¹⁰ In the writer's experiments the rate of respiration began to decrease about 30 minutes after transfer from nutrient solution to distilled water.

plastic material is used in oxidation. If this is true it is easily conceivable that the oxidation of such material might not be affected in the same way by CaCl_2 as the oxidation of dextrose.

Experiments on antagonism between NaCl and CaCl_2 were also made. A solution containing 19 cc. of NaCl and 1 cc. of CaCl_2 (both 0.5M) gave the best results. In this mixture the rate of respiration was practically normal (Fig. 3, Curve C). Other proportions gave more or less increase.

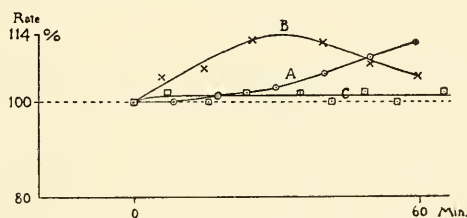


FIG. 3. Respiration of *Aspergillus niger*. The broken straight line to the left of the point marked 0 on the abscissa represents the normal rate of respiration in 0.1 per cent dextrose before the addition of the salt. Curve A represents the respiration in 0.5M NaCl , Curve B in 0.5M CaCl_2 , and Curve C in a mixture of 19 cc. of NaCl and 1 cc. of CaCl_2 (both 0.5M). The control (dotted line) and C are practically identical. All these experiments were made in 0.05 per cent dextrose. Curve A is an average of 3, Curves B and C of 4 experiments. The probable error was less than 3 per cent of the mean.

In order to compare these results with the effects on growth spores were sown on solutions of NaCl and CaCl_2 (with 0.05 per cent dextrose), on various mixtures of these,¹¹ and on 0.05 per cent dextrose alone. For these experiments Petri dishes were used. The extent of germination was determined by microscopic examination.

The following results were noted: In NaCl there was no germination; in 49 cc. of NaCl +1 cc. of CaCl_2 some germination; 24 cc. of NaCl +1 cc. of CaCl_2 , and 9 cc. of NaCl +1 cc. of CaCl_2 showed a growth which increased in proportion to the amount of CaCl_2 ; 4 cc. of NaCl +1 cc. of CaCl_2 seemed to produce the best growth; 1 cc. of NaCl +1 cc. of CaCl_2 and CaCl_2 alone showed fairly good growth; 0.05 per cent dextrose produced rather poor growth.

¹¹ Both NaCl and CaCl_2 were 0.5M and were dissolved in 0.05 per cent dextrose.

From these data it would seem that effects of CaCl_2 and NaCl on respiration are different from their effects on growth. This difference cannot be an osmotic effect, as the CaCl_2 solution, having a stronger osmotic pressure, would in that case be the one that would prevent growth to a greater extent, but this is not the fact.

SUMMARY.

1. In the presence of 0.05 per cent dextrose the respiration of *Aspergillus niger* is increased by NaCl in concentrations of 0.25 to 0.5M, and by 0.5M CaCl_2 .
2. Stronger concentrations, as 2M NaCl and 1.25M CaCl_2 , decrease the respiration. The decrease in the higher concentrations is probably an osmotic effect of these salts.
3. A mixture of 19 cc. of NaCl and 1 cc. of CaCl_2 (both 0.5M) showed antagonism, in that the respiration was normal, although each salt alone caused an increase.
4. Spores of *Aspergillus niger* did not germinate on 0.5M NaCl (plus 0.05 per cent dextrose) while they did on 0.5M CaCl_2 (plus 0.05 per cent dextrose) and on various mixtures of the two. This shows that a substance may have different effects on respiration from those which it has upon growth.

THE RELATIVE PHYSIOLOGICAL EFFECTS OF β - AND γ -RAYS UPON THE EGG OF NEREIS.

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(Received for publication, June 17, 1919.)

As a preliminary to an investigation of the relative effects of β -rays of different velocities upon the egg of the marine worm, *Nereis limbata*, it is desirable to determine how far the reaction in question is due to the β - and to the γ -rays.¹ This information is also of importance in connection with a recently published² determination of the temperature coefficient of the action of the rays from radium upon these eggs since, as will be seen, it enables us to assign this coefficient to the action of the β -rays.

When unfertilized *Nereis* eggs are exposed to radiations from radium and then fertilized, the fertilization membrane which results is of unusual thickness.³ We have shown that this reaction may be made to serve as a measure of radioactivity since a simple relation exists between the volume of the membrane formed by the eggs and the intensity of the radiation to which they have been exposed.⁴ When used in this way the *Nereis* egg will give a measure of the intensity of the rays only in as far as they are physiologically effective. Our method has consisted in exposing a series of eggs for uniform periods of time to radiations passing through various thicknesses of aluminium and in determining the resulting membrane volumes. At the same time a series of eggs from the same female worm was exposed to various known intensities of radiation, produced by varying the distance between the eggs and the source of radiation, and evalu-

¹ Redfield, A. C., and Bright, E. M., *J. Gen. Physiol.*, 1919-20, ii, 31.

² Redfield, A. C., and Bright, E. M., *J. Gen. Physiol.*, 1918-19, i, 255.

³ Packard, C., *J. Exp. Zool.*, 1915, xix, 323.

⁴ Redfield, A. C., and Bright, E. M., *Am. J. Physiol.*, 1917-18, xlv, 374.

ated according to the formula of Wood and Prime.⁵ A curve was then plotted relating the intensity of radiation to the membrane volumes resulting. This curve was used to determine the intensities of radiation reaching the first series of eggs through the various thicknesses of aluminium.

From data obtained in this way a coefficient could be obtained expressing the absorption of the physiologically effective radiation by aluminium. In our experiments the radioactive source has consisted of radium emanation, in equilibrium with its products, contained in a slender glass tube, the walls of which were sufficiently thick to absorb all the α -rays.⁶ Such a preparation emits β - and γ -rays. The former are much less penetrating than the latter and possess as a result a much higher coefficient of absorption. If the physiological effect is due wholly or in large part to one of these types of radiation to the exclusion of the other, the absorption coefficient of the physiologically effective radiation should be of the same magnitude as the absorption coefficient of that type of radiation. If, on the other hand, both sorts of radiation contribute considerably to the physiological reaction, the absorption coefficient determined by it should have an intermediate value. Fig. 1 shows the results of three experiments. Against the thickness of the aluminium filters are plotted the logarithms of the per cent of the radiation passing through the filters, estimated from the effect upon *Nereis* eggs. Through these points we have drawn a line which corresponds to an absorption coefficient, μ , of 23.9 cm.^{-1} estimated according to the equation

$$\frac{I}{I_0} = e^{-\mu d}$$

where I is the intensity of radiation passing through a thickness of aluminium d , and I_0 is the intensity of the original beam.

The β -radiation from preparations such as we have employed is due chiefly to the disintegration of Radium *B* and *C*. The velocity

⁵ Wood, F. C., and Prime, F., Jr., *Ann. Surg.*, 1915, lxii, 751.

⁶ We are greatly indebted to Dr. William Duane and to Dr. R. B. Greenough, the Director of the Cancer Commission of Harvard University, for placing a supply of radium emanation at our disposal.

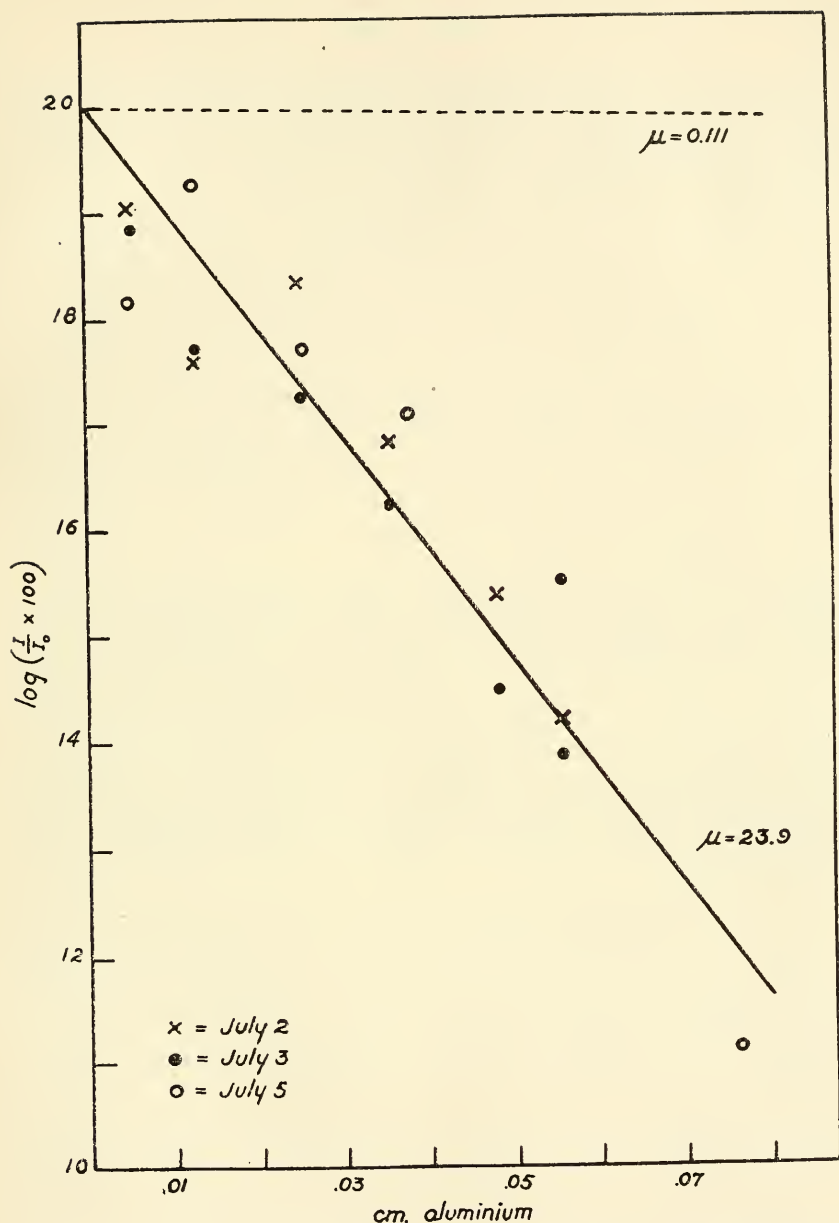


FIG. 1. Absorption of radiations from radium by aluminium measured by their effect upon the egg of *Nereis*. Thickness of aluminium measured in cm. along the abscissa. Per cent of transmitted radiation measured logarithmically along the ordinate. The dotted line indicates the absorption of γ -rays.

of these rays and their consequent absorption coefficients are not uniform. The following values may be assigned to the absorption coefficients of these rays.⁷

Substance.	μ cm.^{-1}
Radium B	$\left\{ \begin{array}{l} 13.1 \\ 80.0 \\ 890.0 \end{array} \right.$
“ C	$\left\{ \begin{array}{l} 13.2 \\ 53.0 \end{array} \right.$

In contrast to these figures the absorption coefficient of the γ -rays is of a smaller order, being 0.111 according to Soddy and Russell.⁸ The dotted line in Fig. 1 indicates how little the filters used would cut down the intensity of rays with this coefficient of absorption. If the absorption coefficient, $\mu = 23.9 \text{ cm.}^{-1}$, for the physiologically effective radiation is compared with these values it is obvious that this figure is of the order of magnitude of the absorption coefficient of β -rays. We conclude, therefore, that the β -rays constitute the physiologically effective radiation and that the influence of the γ -rays on this reaction is negligible. This result is quite in accordance with expectation, for a number of investigations have indicated that the physiological effects of radiations of different types are roughly proportional to the ionizing powers of the rays involved.⁹ The ionizing power of the γ -rays is probably about only 1 per cent of that of the β -rays.

In order to determine whether the γ -rays have any effect on the volume of the fertilization membrane of the *Nereis* egg a lot of eggs was exposed for 11.5 hours to an intensity of radiation of 65 millicurie centimeters filtered through 1.9 mm. of lead and 1.7 mm. of cardboard. This thickness of lead completely absorbs the primary β -radiation while the cardboard stops the slow electrons sent out from the lead by the γ -rays. The membranes of eggs treated in this way had an average volume of 5.9×10^5 cubic microns, while

⁷ Rutherford, E., *Radioactive substances and their radiations*, Cambridge, 1913, 225.

⁸ Soddy, F., and Russell, A. S., *Phil. Mag.*, 1909, xviii, 620.

⁹ Russ, S., *Arch. Middlesex Hospital*, 1912, xxvii, 16. Lazarus-Barlow, W. S., and Beckton, H., *ibid.*, 1913, xxx, 47.

the average volume of a control of unirradiated eggs was only 1.5×10^5 cubic microns. This result would indicate that the γ -rays contribute to the reaction of the *Nereis* egg. How small this contribution must be, however, is indicated by the fact that an exposure of about 11 minutes to 65 millicurie centimeters of β - and γ -radiation will produce an equivalent change in the membrane volume. The γ -rays alone require an exposure 60 times as long as the β - and γ -rays together. Inasmuch as long exposures to low intensities produce a greater effect upon these eggs than short exposures to high intensities,⁴ the intensity of the physiologically effective β -rays must be much more than 60 times as great as the intensity of the physiologically effective γ -rays.

SUMMARY.

When *Nereis* eggs are exposed to radiations from a tube of radium emanation, the walls of which absorb all the α -rays, the resulting physiological change is produced by rays having a coefficient of absorption of the order of 23.9 cm.^{-1}

This fact indicates that the physiological effect is due almost exclusively to β -rays.

The γ -rays alone can produce the reaction. To produce equivalent physiological effects exposure to γ -rays alone must be approximately 60 times as long as exposure to β - and γ -rays together.

THE RELATIVE PHYSIOLOGICAL EFFECTS OF β -RAYS OF DIFFERENT VELOCITIES.

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(Received for publication, June 17, 1919.)

Insight into the nature of the action of any reagent upon protoplasm can be gained only through the correlation of its physical and chemical properties with its physiological effects. The present investigation is an attempt to discover whether any quantitative relation exists between the physiological effects of β -rays from radium of different velocities and their physical characteristics.

At the present time it is impossible to state definitely whether qualitative differences exist in the reactions of tissues to α -, β -, γ -, and x-rays¹ although the characteristic effects in accelerating and retarding cell division and growth appear to be the same.^{2, 3} It is also indicated that the magnitude of the physiological action of these various kinds of rays corresponds roughly with their strength, as measured by their action in ionizing air.⁴

Several investigators have already undertaken to compare the effects of slow and fast β -rays from radium, but none of them have attempted to procure homogeneous groups of rays or to determine more than relatively the velocities of the rays used. Packard, who by means of a strong magnetic field and with the aid of mica filters

¹ For evidence of specific effects of different types of rays see Beckton, H., *Arch. Middlesex Hospital*, 1914-15, xxxii, 123. Chambers, H., and Russ, S., *ibid.*, 1912, xxvii, 29. Colwell, H. A., and Russ, S., Radium, x-rays and the living cell, London, 1915, 146.

² Lazarus-Barlow, W. S., and Beckton, H., *Arch. Middlesex Hospital*, 1913, xxx, 47. Packard, C., *J. Exp. Zool.*, 1916, xxi, 199. Richards, A., *Biol. Bull.*, 1914, xxvii, 67.

³ Packard, C., *J. Exp. Zool.*, 1915, xix, 323.

⁴ Russ, S., *Arch. Middlesex Hospital*, 1912, xxvii, 16. Lazarus-Barlow, W. S., and Beckton, H., *ibid.*, 1913, xxx, 47.

separated the β -rays, observed certain differences in their effects upon the eggs of *Arbacia* and *Nereis*, but in this investigation no attempt was made to compensate for the differences in intensity of the rays to which the cells were exposed.³ Congdon compared the effects obtained by exposing seeds to a primary beam of β -rays with the effects of the same beam plus the soft rays, which were scattered back from the primary beam from a surface of lead, and concluded that in proportion to their energy content the slower rays have a greater retarding action than the more rapid rays.⁵

The development of a method of measuring the physiological action of radiations from radium has enabled us to examine the problem quantitatively. The method depends upon the fact first observed by Packard that the fertilization membranes of the eggs of the marine worm, *Nereis limbata*, are greatly enlarged if the eggs have been exposed to radium prior to fertilization.³ We have shown that the extent of this change is a reliable measure of the intensity of radiation.⁶ When used as instruments for measuring the strength of a beam of heterogeneous rays, the eggs will take account of each ray only in as far as it is effective physiologically. We have shown that the influence of the γ -rays upon these eggs is negligible when compared to the β -rays,⁷ and have consequently neglected their consideration in interpreting our experimental results. The general plan of the investigation has been to expose *Nereis* eggs to approximately homogeneous beams of β -rays of various velocities, to compare the effects so produced with the effects produced on eggs from the same worm by a heterogeneous beam of β -rays of known intensity, and in this way obtain a measure of the relative *physiologically effective radiation* in each homogeneous beam. These values have then been compared with the relative strengths of each homogeneous beam as measured by their ability to ionize air. Before going into the details of the experiments it may be stated that they indicate that the ratio of the physiological effect and the ionizing power of β -rays is constant for all velocities.

⁵ Congdon, E. D., *Arch. Entwcklgsmechn. Organ.*, 1912, xxxiv, 267.

⁶ Redfield, A. C., and Bright, E. M., *Am. J. Physiol.*, 1917-18, xlv, 374.

⁷ Redfield, A. C., and Bright, E. M., *J. Gen. Physiol.*, 1919-20, ii, 25.

Procedure.

Homogeneous groups of β -rays have been procured by the method commonly used by physicists, which depends on the fact that in traversing a magnetic field these negatively charged bodies are deflected in a direction normal to the field and as a consequence travel in curved paths. The relation between the velocity of a β -particle, u , the radius of curvature of its path, R , and the strength of the magnetic field, H , are given by the equation

$$HR = \frac{m u}{e}$$

where m is the mass of the particle and e its charge. For our purpose it will suffice to note that the relative velocity of the β -ray is measured by HR , the product of the radius of curvature and the strength of the magnetic field, which are predetermined experimental conditions. By making the usual assumptions concerning the value of e and of m for various velocities absolute values might be assigned to the velocities of the beams of β -rays which we have used.

The apparatus which we have employed is illustrated in Fig. 1. A solenoid, 50 cm. long, was wound about a hollow water jacket, *WJ*. Knowing the number of turns of wire in the solenoid, its length, radius, and the strength of current passing through it, the strength of the magnetic field, H , in its center could be calculated directly. A stream of water circulating through the water jacket prevented the heat generated in the solenoid from increasing the temperature of the space within. Into this space could be introduced an arrangement for exposing the eggs to rays separated into homogeneous groups by the magnetic field. This device consisted of two lead blocks so arranged that a slender glass tube containing radium emanation, E , was supported 1 cm. from a slit, S , through which a beam of rays escaped.⁸ The width of the slit was 0.1 cm. in some experiments, 0.065 cm. in others. When undeflected, the rays leav-

⁸ We are greatly indebted to Dr. William Duane for indispensable advice in designing our apparatus, and to Dr. R. B. Greenough, the Director of the Cancer Commission of Harvard University, for placing a supply of radium emanation at our disposal.

ing this slit passed on through a distance of 2 cm. and impinged at *A* upon a glass plate on which the eggs were spread. In the presence of a magnetic field a slow ray, travelling in a course having a small radius of curvature, would strike the glass plate at *C* while a fast

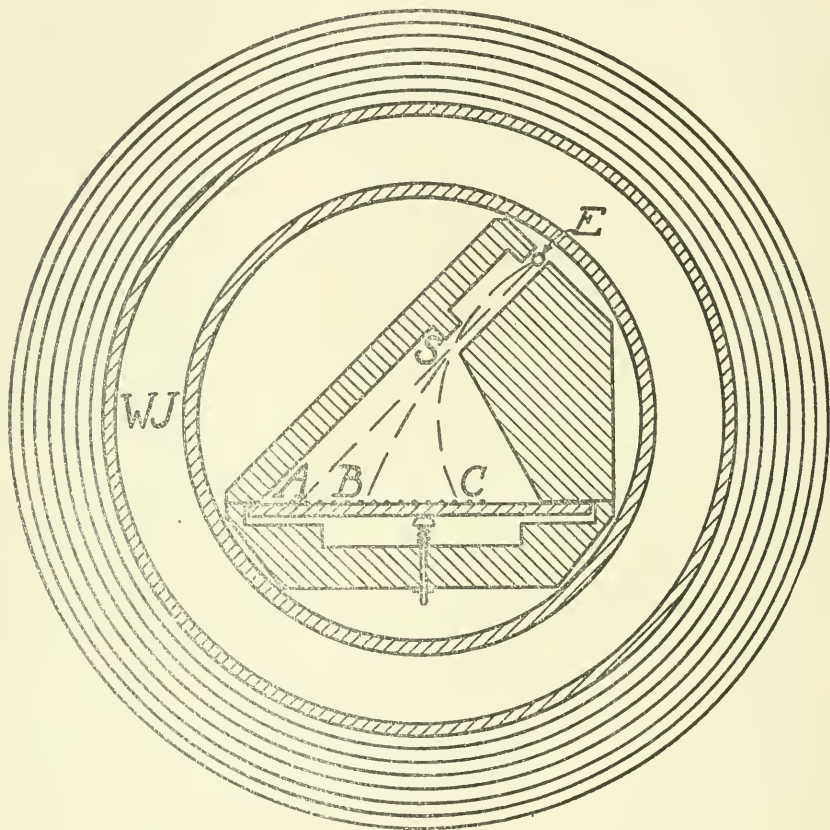


FIG. 1. Diagram of apparatus for exposing *Nereis* eggs to homogeneous groups of β -rays of different velocities.

ray travelling more nearly in a straight line would strike at *B* nearer to *A*. Fig. 2 is a photographic record of the course of the direct and deflected beam.

Two procedures were employed to expose eggs to beams of rays of different velocities. In one the magnetic field, *H*, was kept con-

stant while the radius of curvature, R , was varied by placing the eggs at different positions on the glass plate. In the other the magnetic field was varied by altering the current flowing through the solenoid, while the radius of curvature was kept constant by placing each lot of eggs in the same position on the glass plate.

In the former procedure eggs were spread uniformly over the plate in a thin film of sea water and radiated for 1 or 2 hours in the presence of a strong magnetic field.⁹ Slender filaments of glass cemented across the plate divided it into chambers, each at a measured distance from A , and prevented the eggs from moving from the position which

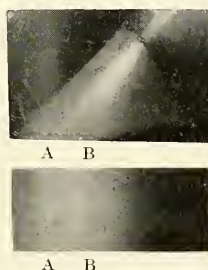


FIG. 2. Photograph of the course of the β -rays when undeflected, A , and deflected, B , by a magnetic field of about 450 gauss. The upper picture was made by placing the photographic plate against the end of the lead blocks which define the beam, so that it was struck a glancing blow by the rays. The lower picture was made by placing the photographic plate under the lead blocks in the position occupied by the glass plate which supported the eggs.

they occupied while being radiated. At the completion of the period of radiation the plate was removed from the apparatus and the eggs were fertilized by blowing a shower of sperm suspended in sea water upon them with an atomizer. This procedure was essential in order to prevent eggs being washed from one compartment on the plate to another. About 40 minutes after fertilization, a cover-glass was laid across the glass filaments, which protected the eggs from being crushed, and measurements of the thickness of the membranes of

⁹ Preliminary experiments established the fact that exposure to the magnetic field alone had no effect upon the membrane of the *Nereis* eggs.

the eggs were begun. In making these measurements one egg from each compartment was measured in turn, then a second from each compartment, then a third, and so on until ten or twelve eggs from each compartment had been measured, by which time the eggs had begun to divide and it was impossible to obtain further exact measurements. In this way we were assured that the figures for each compartment were made the same average time after fertilization, a matter of much importance. This procedure had the advantage that all the eggs were radiated at one time after they had been freshly removed from the female worm. Its disadvantage lay in the fact that it was impossible to measure very many eggs from each lot since all the lots had to be measured at once.

The alternative procedure consisted in placing successive lots of eggs in the same position on the glass plate so that each lot was exposed to rays travelling through the same path. In this case R , the radius of curvature of the effective beam, remained constant. Each lot of eggs was exposed for an equal period of time, 1 or 2 hours, but with a different amount of current flowing through the solenoid, so that the strength of the magnetic field, H , varied. At the termination of the period of radiation each lot of eggs was washed into a watch-glass of sea water, fertilized, and after a uniform period the membranes of twenty-five eggs were measured. This procedure possessed the advantage that a larger number of eggs from each lot could be measured, but it was so time-consuming that only a limited number of homogeneous beams could be measured in each experiment. Moreover, as the experiment proceeded measurements were necessarily made with eggs which had been removed from the female worm for longer and longer periods. We have some reason to suspect that these eggs become slightly less sensitive to radiation after they have stood in sea water for a long period.

The figures obtained from such experiments as these give a measure of the amount of physiological effect from each beam of homogeneous radiation. We have shown, however, that the amount of physiological effect, as indicated by the volume of the fertilization membrane of *Nereis* eggs, is not a direct measure of the intensity to which they have been exposed. What we desire is a measure of the physiologically effective radiation in each group of homogeneous rays which

can be compared with a measure of the same beam obtained by some standard physical method. Since we have shown that the volume of the membrane is a direct function of the logarithm of the intensity of radiation through a considerable range of membrane volumes, it is a simple matter to calibrate each lot of eggs so that the intensity of radiation can be approximated from any known membrane volume.⁹ To do this a series of measurements was made of the physiological effects of various intensities of the entire radiation from a tube of radium emanation, the intensity being determined by the distances between the tube and the eggs according to the formula of Wood and Prime.¹⁰ From data obtained in this way a curve, such as that shown in Fig. 3, was drawn relating the intensity of radiation with the resulting membrane volume. This curve was then used to determine the relative intensity of physiologically effective radiation in each beam of homogeneous β -rays by finding on the curve the intensity which corresponds to the average volume of the membranes of each lot of eggs.

Having obtained in the preceding manner a measure of the relative intensity of the physiologically effective radiation in each beam of β -rays of homogeneous velocity it became necessary to determine the total intensity of each beam by some standard physical method. Although the relative number of β -particles of various velocities given off by tubes of radium emanation in equilibrium with its products has been determined with precision,^{11, 12} two considerations made it desirable to obtain this information with the apparatus used for the physiological determinations. In the first place the slit, S , used to separate the rays into homogeneous beams was of finite dimensions so that it was only possible to obtain an approximation to homogeneity. Since our physiological method of measurement was relatively insensitive it was necessary to keep the distances through which the rays travelled short and the slit wide in order that the effect upon the eggs might be great enough to be measured accurately. Another source of error in the method was due to the fact that a considerable

¹⁰ Wood, F. C., and Prime, F., Jr., *Ann. Surg.*, 1915, lxii, 751.

¹¹ Wilson, W., *Proc. Roy. Soc. London, Series A*, 1909, lxxxii, 612.

¹² Wilson, W., *Proc. Roy. Soc. London, Series A*, 1911, lxxxv, 240.

number of rays struck the edges of the slit and were "reflected" in all directions so that all the beams were contaminated by a considerable number of rays of velocity considerably greater or smaller than the modal velocity of the beam. The only way the contribution of these scattered rays to each beam could be accounted for was a direct physical measurement made with the same apparatus as was used for the physiological experiments.

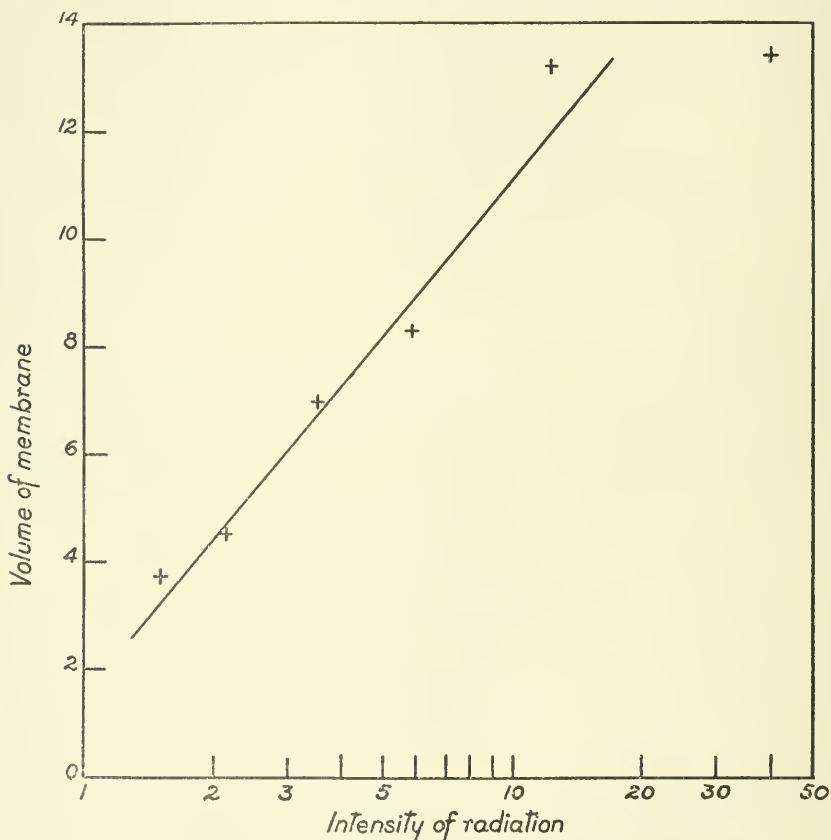


FIG. 3. Curve used to determine the relative intensity of the physiologically effective radiation from its effect on the membrane of *Nereis* eggs. The points are experimental results obtained with known intensities of heterogeneous radiation. The line is the curve used in calibrating the experimental results with homogeneous beams. Intensities measured in millicurie centimeters along the abscissa. Membrane volumes measured in 100,000 cubic microns along the ordinate. Experiment of August 29, 1918.

In order to calibrate the "spectrum" of β -rays the apparatus was modified as shown in Fig. 4. To make more room the water jacket was removed from the interior of the solenoid, and a brass tube arranged to extend into the center of the coil and support a small ioni-

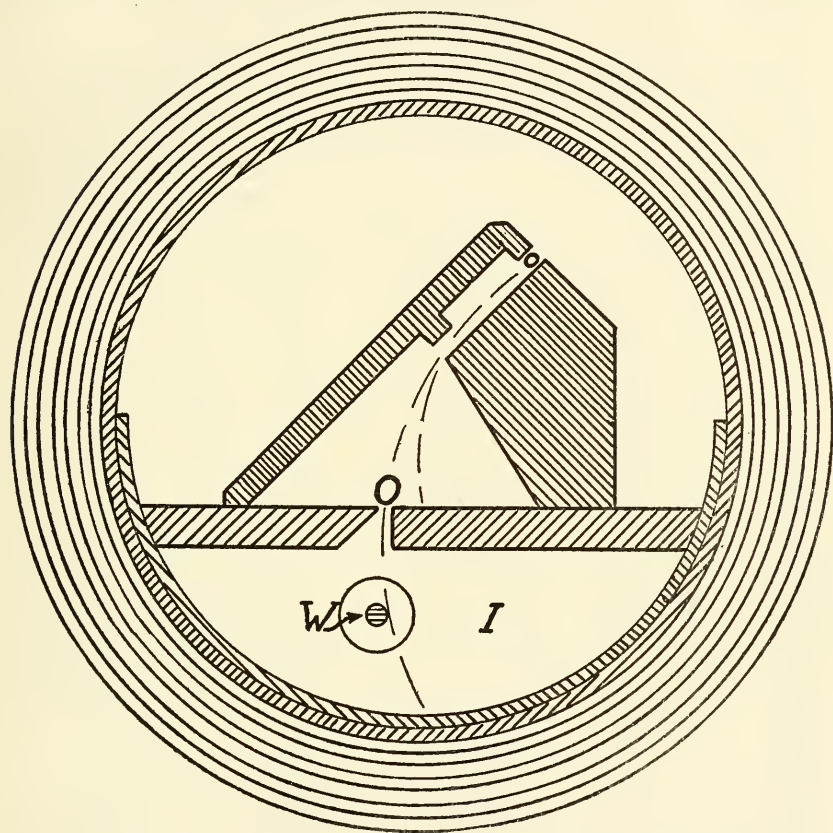


FIG. 4. Diagram of arrangement used to determine the relative ionizing powers of homogeneous groups of β -rays to different velocities.

zation chamber, *I*. The roof of this chamber consisted of a lead plate 3 mm. thick which prevented any β -rays from entering the chamber except through a hole, *O*, 1 mm. wide and 1 cm. long. The lead blocks which supported the tube of radium emanation rested

upon the roof of the ionization chamber, and could be slid back and forth so that a beam of β -rays of any desired radius of curvature entered the hole. When these rays passed through the ionization chamber the air in it became ionized. The relative number of ions formed by the various beams of homogeneous rays was measured by the rate at which they conducted electricity between the wall of the ionization chamber, charged to 120 volts, and a suitably insulated wire, W , which projected into the chamber from one end. This wire led to a quadrant electrometer arranged as described by Duane for measuring ionization currents of this sort.¹³ Several corrections had to be applied to the measurements of ionization made in this way. A considerable part of the ionization current was due to the effects of γ -rays which passed through all parts of the lead roof of the ionization chamber and to secondary β -radiations set up by them on striking the walls of the chamber. A smaller increment was due to the leak of electricity across the insulation of the wire. To correct for these the roof of the ionization chamber was replaced by a piece of lead in which there was no hole and a measurement made which expressed the ionization due to the γ -rays and the leak. This value subtracted from the former measurement, gave an expression of the amount of ionization due to the β -rays alone. As the air within the ionization chamber became warm from the heat generated in the coil its density became less and as a result the number of ions formed by a given beam of rays was reduced in proportion to the rise in the absolute temperature. Care was taken to record the temperature of the air within the solenoid at the time each measurement was taken and to correct each reading to a standard temperature, for which 27°C . was chosen. In addition a slight correction was made for the decay of the radium emanation in the course of the experiment. By these procedures figures were obtained expressing in arbitrary units the relative ionization produced by each of the beams of β -rays which had been measured physiologically, and under exactly the same conditions.

¹³ Duane, W., *Boston Med. and Surg. J.*, 1917, clxxvii, 787.

Results.

Seventeen experiments were conducted on *Nereis* eggs with these methods. Of these nine of a preliminary nature were rejected either because insufficient data were obtained for determining the effective

TABLE I.

Nereis Eggs Exposed to Homogeneous Beams of β -Rays Separated by a Uniform Magnetic Field but Travelling through Paths of Various Radii of Curvature.

July 9, 1918. $H = 477$ gauss. R variable. 0.1 cm. slit. Strength of emanation = 46 millicuries. Time of radiation = 90 min. Twelve eggs measured in each lot.

HR	Volume of membrane.	Relative effective radiation.	Relative ionization.	Ionization. Effective radiation.
<i>gauss cm.</i>	$10^5 \mu^3$			
840	4.40	19.8	20.0	1.01
965	4.50	23.5	23.0	0.98
1,120	6.10	28.1	44.0	1.57
1,315	7.90	49.1	62.0	1.26
1,595	11.60	82.5	80.0	0.97
1,990	12.55	98.5	96.5	0.98
2,600	12.65	100.0	100.0	1.00
3,445	11.60	82.5	95.5	1.16
5,010	10.90	73.6	78.0	1.06

Aug. 21, 1918. $H = 502$ gauss. R variable. 0.065 cm. slit. Strength of emanation = 58.4 millicuries. Time of radiation = 120 min. Eight to twelve eggs measured in each lot.

932	6.00	48.0	35.4	0.74
1,070	6.15	50.0	46.1	0.92
1,235	6.35	51.7	62.2	1.20
1,475	6.50	54.7	80.8	1.48
1,805	7.30	67.4	97.3	1.44
2,250	8.65	100.0	100.0	1.00
3,015	8.05	81.6	91.5	1.12
4,230	7.90	77.5	72.5	0.94
6,560	5.80	45.0	47.8	1.06

intensity from the physiological change obtained, or because too few determinations were made, or because the change in the membrane volumes obtained were too small for accurate measurement. One experiment was rejected because the measurements obtained were

TABLE II.

Nereis Eggs Exposed to Homogeneous Beams of β -Rays Separated by a Variable Magnetic Field but Travelling through Paths of the Same Radius of Curvature.

Aug. 1, 1918. H variable. $R = 8.45$ cm. 0.065 cm. slit. Strength of emanation = 51.5 millicuries. Time of radiation = 60 min. Twenty-five eggs measured in each lot.

HR	Volume of membrane.	Relative effective radiation.	Relative ionization.	Ionization. Effective radiation.
<i>gauss cm.</i>	$10^5 \mu^3$			
0	3.95	49.0	53.5	1.09
1,070	5.61	89.5	85.0	0.95
1,510	5.71	92.0	96.0	1.04
2,265	6.30	100.0	97.5	0.98
3,060	5.71	92.0	87.0	0.95
4,220	5.50	84.5	68.0	0.81

Aug. 29, 1918. H variable. $R = 13.1$ cm. 0.065 cm. slit. Strength of emanation = 70 millicuries. Time of radiation = 90 min.

0	3.70	55.0	76.6	1.39
1,560	6.05	100.0	100.0	1.00
2,860	5.80	93.5	96.5	1.03
4,100	5.70	91.5	82.8	0.91
5,340	4.10	62.5	66.7	1.07
6,570	4.85	75.5	57.5	0.76
7,320	3.35	51.0	52.5	1.03

Aug. 29, 1918. H variable. $R = 8.45$ cm. 0.065 cm. slit. Strength of emanation = 70 millicuries. Time of radiation = 90 min.

0	2.90	46.0	53.5	1.16
1,010	5.20	81.0	83.6	1.03
1,845	5.80	92.0	100.0	1.09
2,645	6.10	100.0	93.0	0.93
3,440	4.75	72.0	78.6	1.09
4,240	5.60	88.5	68.2	0.77
5,040	4.00	60.0	55.0	0.92

Aug. 30, 1918. H variable. $R = 7.65$ cm. 0.01 cm. slit. Strength of emanation = 60 millicuries. Time of radiation = 75 min.

0	4.35	38.2	35.2	0.92
531	5.55	53.6	48.0	0.90
1,305	5.60	54.0	72.0	1.33
2,045	8.50	88.6	95.0	1.07
2,765	9.80	105.0	100.0	0.95
3,485	9.10	94.8	98.7	1.04
4,210	9.30	99.0	90.2	0.91
4,925	6.60	64.0	81.5	1.27

TABLE II—*Concluded.*

Aug. 30, 1918. H variable. $R = 5.45$ cm. 0.01 cm. slit. Strength of emanation = 60 millicuries. Time of radiation = 75 min.

HR	Volume of membrane.	Relative effective radiation.	Relative ionization.	Ionization. Effective radiation.
<i>gauss cm.</i>	$10^5 \mu^3$			
0	3.65	30.2	32.5	1.08
392	4.25	40.7	38.3	0.94
935	4.30	42.0	59.0	1.40
1,405	6.45	70.0	77.1	1.10
2,000	8.70	101.0	92.5	0.92
2,490	8.55	100.0	100.0	1.00
3,000	9.00	104.5	94.5	0.91
3,520	6.70	72.2	91.5	1.27

so irregular that it is our belief that their inclusion would only serve to obscure the truth. Seven experiments remained which we consider satisfactory in every respect and their results are presented in Tables I, II, and III, and Figs. 5 and 6. It may be added that the rejected experiments, as far as can be judged, agree with the data here presented in showing maximum and minimum effects for beams of β -rays of corresponding velocities.

Table I contains the results of two experiments made with the procedure in which the magnetic field, H , remained constant while the radius of curvature, R , of the effective beam of β -rays varied with each lot of eggs. One of these experiments is illustrated graphically in Fig. 5. Table II contains the results of five experiments made with the procedure in which the strength of the magnetic field, H , varied, while the radius of curvature, R , in which the effective beam of β -rays moved was the same for each lot of eggs. One of these experiments is illustrated graphically in Fig. 6. Examination of the tables will show that the intensity of the radiation in each beam as measured physiologically, indicated in the third column of the table, and the amount of ionization produced by each beam in air, indicated in the fourth column, both increase rapidly up to a maximum at about the same value of HR and then fall off again as HR increases further. In the fifth column of these tables the ratio of the relative amount of ionization and the relative physiologically effective radiation is

given. The units in which these values are expressed are arbitrarily chosen so that if a direct relation exists between the ability of β -rays of any velocity to act upon living cells and to ionize air the figures in this column will approach a constant with a value about 1.0. This is seen to be the case. Individual figures fluctuate considerably from this value, as is to be expected in constants derived from physio-

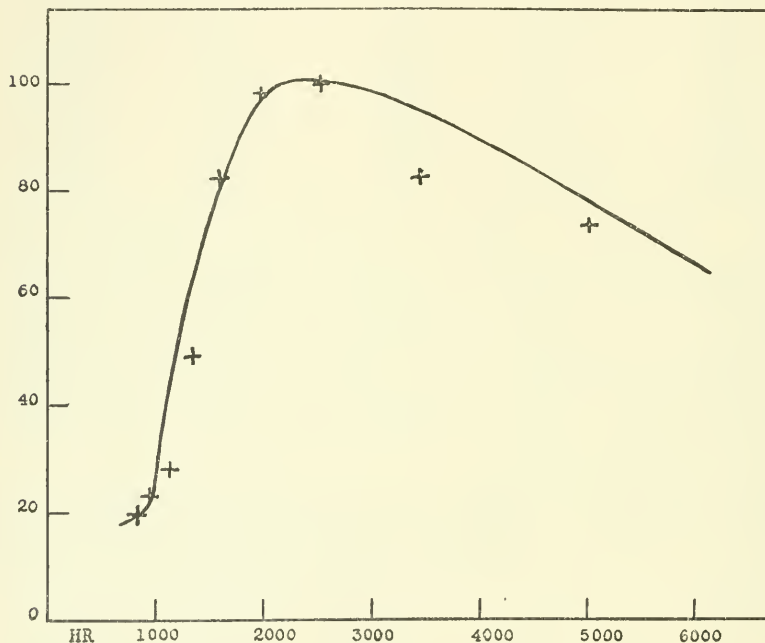


FIG. 5. The solid line represents the relative ionization, measured along the ordinate, produced by β -rays of different velocities, measured along the abscissa. The points represent the relative physiologically effective radiation in the same beams, as determined with *Nereis* eggs. For data see Table I, experiment of July 9, 1918. $H = 477$ gauss. R variable.

logical data, but the deviations do not show any correlation with the velocity of the β -rays under consideration. We have collected all the constants from Tables I and II into groups according to the velocity of the β -rays and have presented the average for each group in Table III. When this is done the fluctuations in the individual constants balance out and it becomes clearly apparent that *as the velocity of*

the rays increases the ratio between their physiological effect and their ionizing power remains constant.

It has been shown by Wilson that a number of slow β -rays produce many more ions per cm. path through air than the same number of fast ones.¹² Since we have shown that there is a constant relation

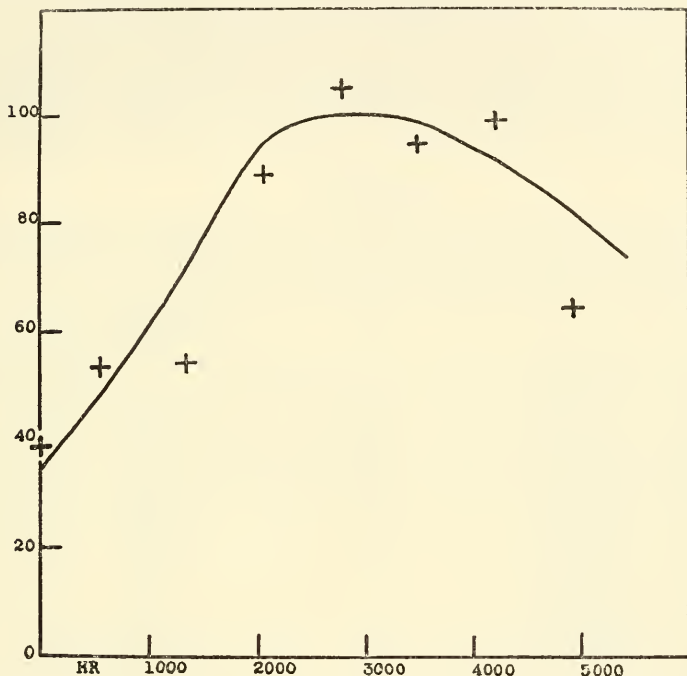


FIG. 6. The solid line represents the relative ionization, measured along the ordinate, produced by β -rays of different velocities, measured along the abscissa. The points represent the relative physiologically effective radiation in the same beams, as determined with *Nereis* eggs. For data see Table II, experiment of August 30, 1918. H variable. $R = 7.65$ cm.

between the ionization produced in passing through the depth of the ionization chamber and the physiological effect produced in passing through the *Nereis* eggs by a β -ray of any velocity, it may be concluded that in passing through a cell a number of slow β -rays will produce much more physiological effect than the same number of fast β -rays.

The finding that the physiological action of the β -rays is closely related to its ionizing power is compatible with the view which has been vaguely suggested by many investigators and definitely formulated by Joly¹⁴ and Richards and Woodward¹⁵ that the physiological effects of x-rays and radiations from radium are due primarily to their ionizing effects upon substances in the protoplasm. It must be remembered, however, that the relative ionization produced by β -rays is probably related to the relative absorption of these rays by matter and our findings might be explained equally well in accordance with the principle of Grotthus. According to this view

TABLE III.

Averages of the Constants in the Fifth Column of Tables I and II Grouped according to the Velocity of the β -Rays Employed.

HR	Ionization. Effective radiation.
<i>gauss cm.</i>	
0- 500	1.13
500-1,000	0.99
1,000-1,500	1.20
1,500-2,000	1.06
2,000-2,500	1.01
2,500-3,000	0.99
3,000-4,000	1.10
4,000-5,000	0.92
5,000-6,000	1.02
6,000-7,000	0.95

slow β -rays produce more physiological change than a corresponding number of fast β -rays because they are absorbed to a correspondingly greater degree. Ionization of some of the constituents of protoplasm need not necessarily be involved in the transformation of the energy consumed in producing the physiological change. To establish the fact that ionization of the cell constituents actually occurs under the influence of these rays, quantitative data of another sort must be forthcoming.

¹⁴ Joly, J., *Proc. Roy. Soc. London, Series B*, 1914-15, lxxxviii, 262.

¹⁵ Richards, A., and Woodward, A. E., *Am. J. Roent.*, 1917, iv, 564.

The conclusion of Congdon will be seen to be at variance with our experimental result.⁵ In his experiments it was observed that the addition of enough soft β -rays to a heterogeneous beam to increase its ionizing power 25 per cent caused an average retardation of 35 per cent in the rate of growth of seeds exposed to the radiations. It is to be regretted that Congdon did not determine how greatly the retardation would have been increased by strengthening the primary beam by 25 per cent without changing its character. If this procedure had yielded retardation of less than 35 per cent we could then feel sure that the discrepancy in our results was a real one.

SUMMARY.

1. The physiological effect upon the eggs of *Nereis* of homogeneous groups of β -rays of different velocities is proportional to their ability to ionize air.
2. β -rays of low velocity produce a greater amount of physiological change than the same number of rays of high velocity.
3. These conclusions are consistent with, but do not prove, the view that the physiological effects of radiations from radium and x-rays are due to the production by them of an ionization of some substance in the egg.

CHANGES IN PROTOPLASMIC CONSISTENCY AND THEIR RELATION TO CELL DIVISION.

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Laboratory, Woods Hole.)

(Received for publication, July 29, 1919.)

I. Periodic Changes in Consistency of the Egg Cytoplasm after Fertilization and during Cleavage.

On fertilization an increase in the viscosity of the semifluid cytoplasm of the sea urchin egg was noticed by Albrecht¹ and recently fully demonstrated by Heilbrunn.² Heilbrunn based his conclusions on his observation that a greater centrifugal force is necessary to stratify the cell constituents of an egg after fertilization than before. I³ have presented evidence, from microdissection studies on the sand-dollar egg and the egg of *Cerebratulus*, that the increase in viscosity is associated with the appearance and growth of the aster.

Upon entrance of the spermatozoon into the egg a diminutive aster makes its appearance as a ball of a jelly-like consistency in the immediate vicinity of the sperm head. This aster, with the sperm nucleus, moves inward as it steadily increases in size until, when its center comes to lie in or near the center of the egg, its radiations extend throughout the whole egg. During this migration the sperm nucleus comes into contact with the egg nucleus. The aster then develops completely around the two nuclei, which fuse to constitute the cleavage nucleus.

The development of the sperm aster in the sea urchin egg is at its height within 10 to 15 minutes after fertilization. This is the

¹ Albrecht, E., Untersuchungen zur Struktur des Seeigeleies, *Sitz-ber. Ges. Morph. u. Physiol.*, 1898, xiv, 133.

² Heilbrunn, L. V., Studies in artificial parthenogenesis. II. Physical changes in the egg of *Arbacia*, *Biol. Bull.*, 1915, xxix, 149.

³ Chambers, R., Jr., Microdissection studies II. The cell aster: A reversible gelation phenomenon. *J. Exp. Zool.*, 1917, xxiii, 483.

time when Heilbrunn informs me he found the egg substance to be of maximum viscosity.

The increase in viscosity of the egg cytoplasm is produced by an influence spreading out in all directions from the center of the aster. While this occurs the central hyaline area of the aster (the hyaloplasmisphere of Wilson) increases in size, and there is strong evidence³ that this is due to the accumulation of a hyaline liquid which separates out of the semisolidifying cytoplasm and flows in very fine converging streams to the center of the aster. It is possible that this and kindred phenomena give to the aster the appearance of radiations from a common center. The consistency of the cytoplasm incorporated in the aster diminishes in firmness on passing from the interior of the aster to its exterior, being greatest in the region bordering on the centrosphere and least at the periphery.

The disappearance of the sperm aster, in the opinion of the writer, occurs through a process of liquefaction. During the liquefaction the substance of the centrosphere collects into two areas at opposite poles of the cleavage nucleus. The experiments to be described in this paper indicate that shortly before cleavage each of these areas becomes a center around which the cytoplasm commences again to pass into a semisolid state. The radial configuration about these areas constitutes the amphiaster. The comparatively firm consistency that the egg now attains for the second time since fertilization is due to two masses, the two asters, instead of to a single aster as was the case shortly after the entrance of the sperm. The importance of this phenomenon in its bearing on cell division is discussed in the last part of this paper.

Experiment 1.—The consistency exhibited by the protoplasm of the sea urchin egg at various periods from the moment of fertilization until the completion of the first cleavage, was ascertained by careful probing with the microdissection needle.

Immediately after fertilization the cytoplasmic granules readily flow by the moving needle. After the sperm has entered the egg, the sperm aster constitutes a comparatively firm mass which gradually increases in size as it moves to a central position in the egg. When the sperm aster is at its full development the highly viscous state of the cytoplasm is detected by the needle. Illustrations of this

are given in a former paper.³ The cytoplasmic granules, instead of being readily dislocated by the moving needle, are held as in a jelly, and movements of the needle produce torsions of the entire egg substance. This condition is at its height 10 to 15 minutes after fertilization.

15 to 20 minutes after fertilization, the radiations of the aster begin to fade from view, with a reversal in the cytoplasm of the semi-solid to a more fluid state. The cytoplasmic granules are now easily dislocated by the moving needle. The more prominent radiations disappear first, while the finer ones persist for some time, owing probably to the viscid nature which the cytoplasm always maintains. The liquid substance of the central hyaline area now flows over the nucleus to its two poles, beyond which it often extends. This causes the appearance characteristic of this stage, of a hyaline streak plainly visible in the otherwise granular cytoplasm of the egg. Toward the end of this stage, which lasts for about 20 to 30 minutes, the hyaline substance finally collects into two semispherical masses lying at the two poles of the nucleus.

Shortly before cleavage, about 40 to 50 minutes after fertilization, an increase in firmness sets in, spreading radially from each of the two centers situated at the poles of the nuclear spindle. This constitutes the amphiaster. The egg elongates, the long axis passing through the two centers of the amphiaster. The cleavage furrow now appears and the egg rapidly divides. The time of appearance of the amphiaster until completion of cleavage lasts from 10 to 15 minutes. The increased viscosity of the egg during this amphiaster stage could be more easily demonstrated by the needle in the eggs of *Echinarachnius* and *Cerebratulus* than in those of *Arbacia*.

After completion of the cleavage process, there are indications that the firmness of the cytoplasm persists in the two blastomeres while they are still more or less spherical. Within 10 to 15 minutes after cleavage the two blastomeres crowd up against one another, each assuming a more nearly hemispherical shape. At this stage their cytoplasm is again quite fluid.

These observations demonstrate a pronounced periodicity in the physical state of the egg subsequent to fertilization and during the first cleavage process. In the immature egg the viscosity is high,

after maturation it drops. Upon fertilization it begins to rise again, to reach its maximum with the full development of the sperm aster. The viscosity drops again and continues low until the approach of cleavage. It thereupon rises again to drop only after completion of the first cleavage. Subsequent to the first cleavage the rhythmic appearance and disappearance of the asters within the blastomeres most probably indicate periodic successions of a process analogous to a jelling and liquefying of the cytoplasm.

The segmentation process may thus be explained as consisting essentially in a growth within the egg of two bodies of material through a gradual transformation of the cytoplasm. This transformation is associated with a change in the physical state of the protoplasm, two semisolid masses growing at the expense of the more fluid portions of the cytoplasm.

II. Cutting Experiments on the Segmenting Egg.

If it is true that the segmenting egg consists of two rather firm masses which are most fluid at their periphery, and if the physical state of the protoplasm is not affected in the process, one should be able to cut a segmenting egg into pieces without disturbing the cleavage plane. Cleavage should, therefore, proceed in such a manner as to complete the separation of what remains of the two bodies within each piece. This is what actually happens. Some experiments of Yatsu,⁴ the results of which he made no attempt to explain, are in full accordance with mine and bear directly on this problem. Yatsu cut the eggs of *Cerebratulus* which were just beginning to segment (anaphase stage) into nucleated and non-nucleated fragments. He found that the cleavage furrow proceeded in its original plane irrespective of whether the fragments were nucleated or not. In Fig. 1, I have diagrammatically presented some of his results. Fig. 1 *a* represents a segmenting *Cerebratulus* egg being cut in a plane parallel to its long axis and to one side of the daughter nuclei. The original furrow persisted in the non-nucleated fragment (*b*) and

⁴ Yatsu, N., Some experiments on cell-division in the egg of *Cerebratulus lacteus*, *Annot. zool. japon.*, 1908, vi, 267.

quickly completed its course in the nucleated fragment (*c*). Somewhat later the cleavage of the non-nucleated fragment (*d*) was also completed. Fig. 1 *e* represents a segmenting egg in which the cut

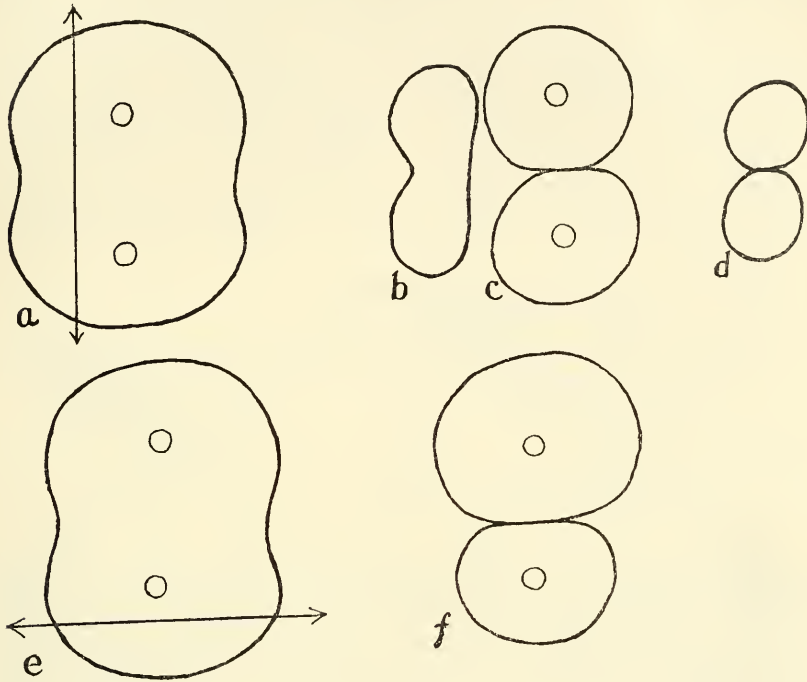


FIG. 1. A diagrammatic representation of Yatsu's results⁴ on cutting the segmenting eggs of *Cerebratulus*. The direction of the cut is shown in *a*. The original cleavage furrow completed its course in the nucleated fragment *c* at the same time that it persisted in the non-nucleated fragment *b*. The furrow finally cut through the non-nucleated fragment in *d*. In *e* a cut was made across one end of the segmenting egg. The original furrow completed its course in *f* resulting in two unequal blastomeres.

was made at one end of the egg at right angles to its long axis. The original furrow persisted so as to divide the mutilated egg into two unequal blastomeres (*f*).

My cutting experiments were carried out mostly on the starfish egg, as sea urchins were very scarce during the summer of 1918.

The mature starfish egg averages 0.16 mm. (*i.e.* 160 μ) in diameter. The needles used for dissection averaged 10 μ in thickness at about 1 mm. from the tip and tapered gradually from there to a point far below 1 μ . With such a needle one can make a puncture or a clean cut through the egg in any desired spot or plane without causing apparent disturbance in the protoplasm of the egg. For cutting purposes glass needles as shown in Fig. 2 were used.⁵ As the egg lies suspended in a hanging drop the end limb of the needle (Fig. 2 *a*) is set in such a way as to push the egg against the cover-slip. Constriction of the egg is produced by a continued upward pressure of the needle until the egg is cut in two. The operation does not neces-

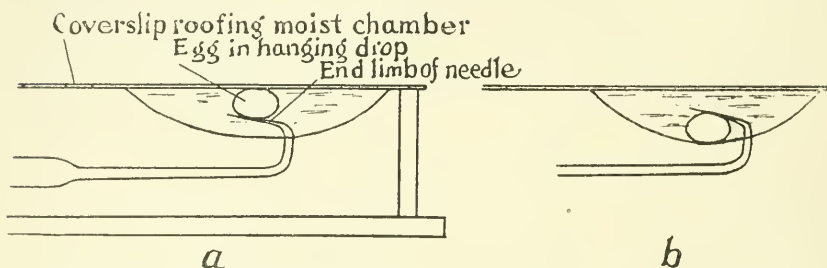


FIG. 2. Methods used for cutting an egg in two. *a*, side view of moist chamber magnified to show needle in position with its end limb so placed as to compress an egg between it and the cover-slip. Continued pressure of the needle cuts the egg in two. *b*, a second method of cutting an egg by bringing the end limb of the needle down on the egg so as to press the egg against the lower surface of the hanging drop.

sarily destroy the fertilization membrane which envelops the egg. The egg may also be cut in two on bringing it (Fig. 2 *b*) between the end limb of the needle and the lower surface of the hanging drop. Lowering the needle out of the drop in such a way as to give to the egg a rolling motion cuts the egg cleanly in two. This second method is not as satisfactory as the first for cases where one wishes to preserve the spatial relations of the egg contents, as the rolling motion produces churning movements within the cell.

Experiment 2.—(Figs. 3 to 7.) An *Asterias* ovum just beginning to segment and with the amphiaster in full development was cut

⁵ Chambers, R., The microvivisection method, *Biol. Bull.*, 1918, xxxiv, 121.

in two in a plane diagonal to the cleavage furrow. The fresh surfaces caused by the cutting form films which prevent reunion of the pieces. The egg was in this way cut into two pieces each consisting of egg substance lying on both sides of the cleavage furrow.

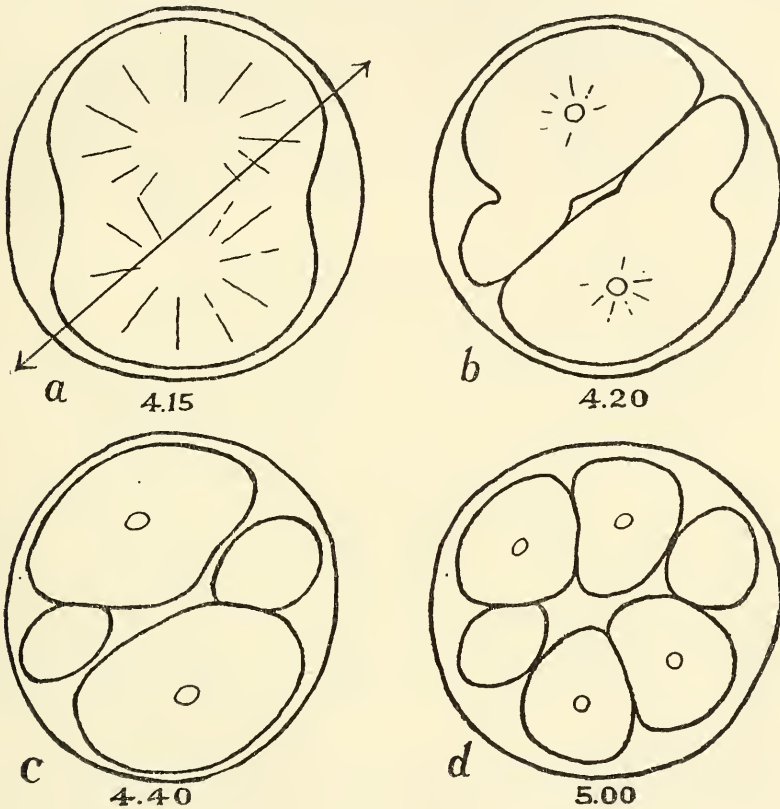


FIG. 3. Effect of a diagonal cut through an *Asterias* ovum beginning to segment in which the cut did not disturb the physical state of the ovum. *a*, operation performed at 4.15 p.m. *b*, 4.20 p.m., persistence of cleavage furrow in the original plane. *c*, 4.40 p.m., non-nucleated fragments pinched off. *d*, 5.00 p.m., nucleated fragments have segmented.

On one occasion the operation was performed on twelve eggs. In nine cases the original cleavage plane was maintained so that each piece pinched off a non-nucleated fragment normally belonging to the other blastomere. Two of them are illustrated in Figs. 3 *a* to *d* and 4 *a* to *d*.

In one case the cut was made at 4.15 p.m. (Fig. 3 *a*). 5 minutes later the cleavage furrow had progressed in the original plane (Fig. 3 *b*). At 4.40 it had completed its course so that each piece was divided into a small non-nucleated and a large nucleated fragment

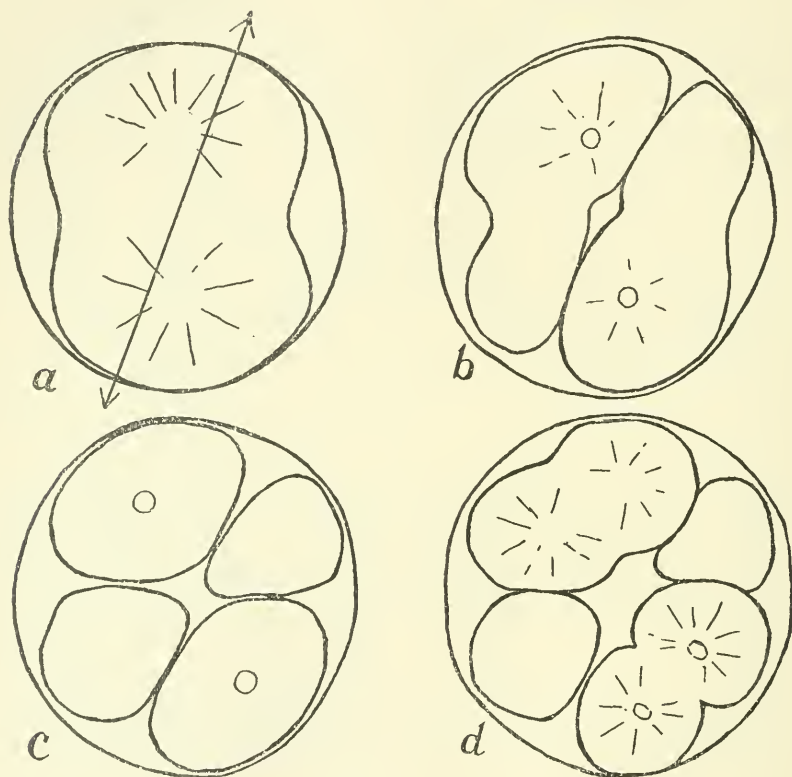


FIG. 4. Similar operation to that shown in Fig. 3 except that the diagonal cut is more nearly perpendicular to the cleavage plane with the result that larger non-nucleated fragments are pinched off by the cleavage furrow.

(Fig. 3 *c*). At 5 p.m. each of the two nucleated fragments or blastomere remnants had divided once (Fig. 3 *d*). 1 hour later they had divided once again. By the next morning the egg developed into a double blastula with the two non-nucleated fragments lying as inert masses within the fertilization membrane.

Fig. 4 *a* to *d* illustrates a similar case in which the non-nucleated masses are considerably larger than those depicted in Fig. 3. The similar behavior of one of the first two blastomeres in an egg is shown in Fig. 5 *a* and *b*.

In the remaining three cases the astral radiations faded out during the operation (Fig. 6 *a*). The original segmentation furrow gradually filled up and disappeared (Fig. 6 *b*) and each piece assumed the appearance of a normal blastomere. The nucleus then shifted so as to occupy a more central position in what one may term the reconstructed blastomere and further segmentation proceeded as if the ovum had not been operated upon (Fig. 6 *c* and *d*). This procedure always

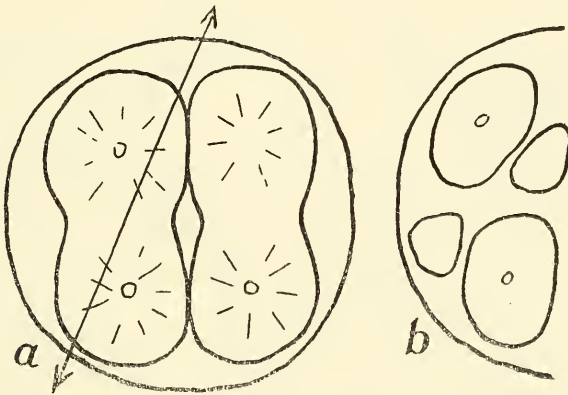


FIG. 5. Effect of a diagonal cut through one of the first two blastomeres of an *Asterias* ovum. *a*, egg showing direction of cut. *b*, cut blastomere a few minutes later.

occurred when the ovum was consciously rolled during the operation so as to produce a disturbance evidenced by a churning movement of the egg constituents.

A similar instance in the case of an *Arbacia* egg is shown in Fig. 7 *a* to *c*. A piece was cut from one pole of the amphiaster egg. In the process the piece was cytolized. The amphiaster in the remainder of the egg disappeared to reappear again in a new position with the result that two equal sized blastomeres were formed.

That mechanical disturbances may cause a reversal of a solid to a fluid state has already been shown.³ This would make all the

protoplasm on each side of the cut merge into a single fluid mass. The nucleus then comes to occupy a central position. Normal mitosis takes place with the formation of an amphiaster and cleavage

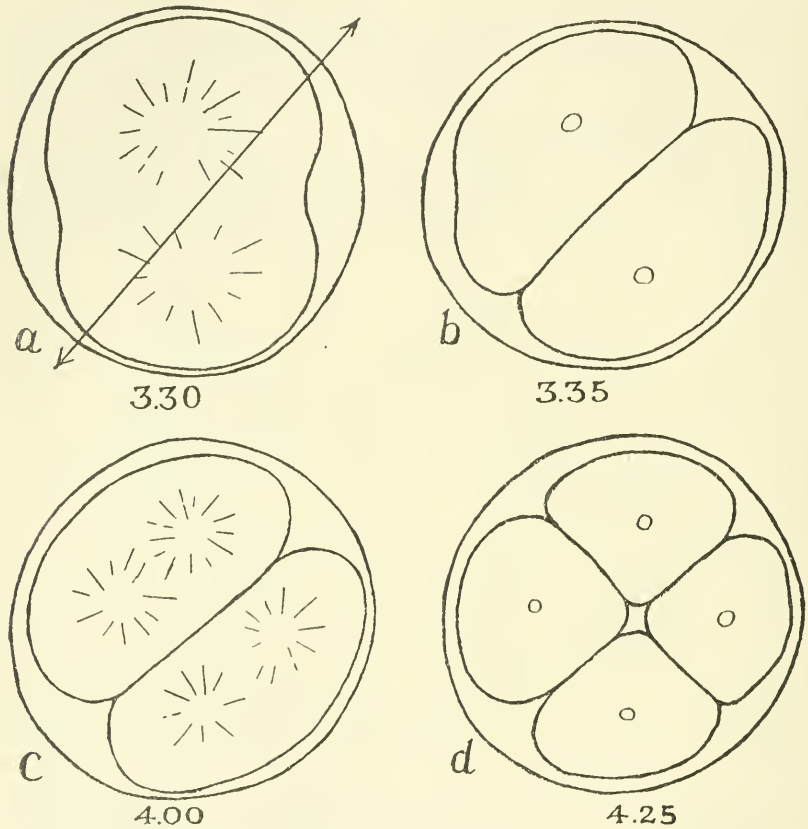


FIG. 6. Effect of a diagonal cut through an *Asterias* ovum in which the cut brought about a change in the physical state of the egg. *a*, operation performed at 3.30 p.m. *b*, 3.35 p.m., original cleavage furrow beginning to be obliterated. *c*, 4.00 p.m., an amphiaster formed in each of the two pieces produced by the cut. *d*, 4.25 p.m., four celled stage in which one cleavage plane was produced by the needle and the other by normal fission.

proceeds along the equator where the boundaries of the two asters are contiguous.

In the nine cases, in which the original cleavage plane persisted after the cutting process, the semisolid state about the two astral

centers was not disturbed. Each of the two pieces resulting from the cut, therefore, consisted of two unequal semisolid masses separated by a fluid area corresponding to the equator of the original egg. As this fluid area is incorporated into the two masses a furrow appears

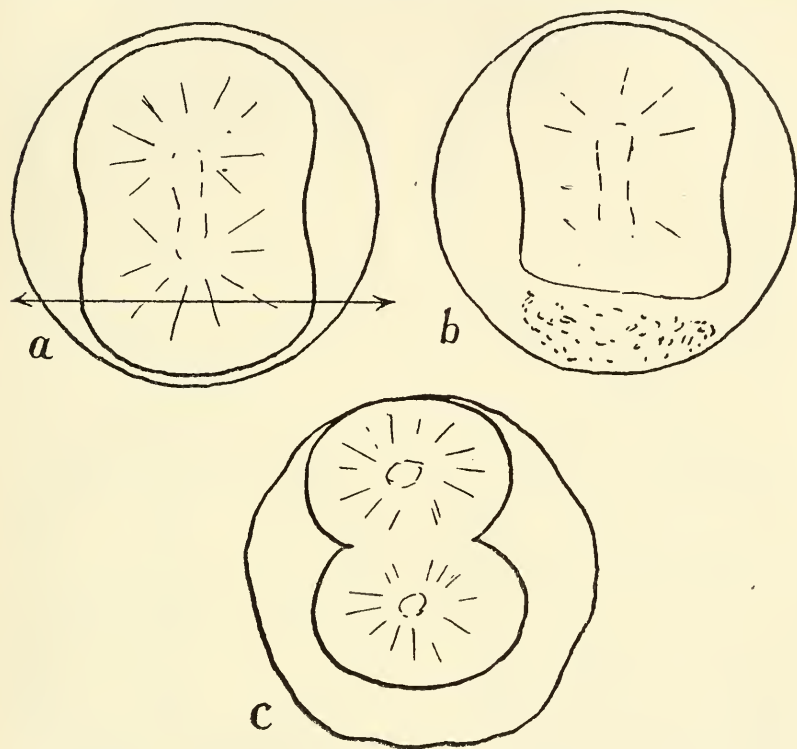


FIG. 7. Effect of cutting off a piece from one pole of an *Arbacia* ovum in the amphiaster stage. *a*, direction of cut. *b*, the piece cut off cytolized. The original shape of the remainder of the egg persisted for some time as the ovum of *Arbacia* is less pliable than that of *Asterias*. *c*, the reappearance of a new amphiaster resulting in the formation of two equal blastomeres.

which separates each piece into a larger nucleated and a smaller non-nucleated body.

The operated eggs were kept under observation until the gastrula stage, indicating that the operation had not destroyed the capacity of the egg for further development.

The following experiments are supplementary to the second. In all of them the results obtained are explicable on the basis of the existence, during cleavage, of reversible changes in the consistency of the cytoplasm.

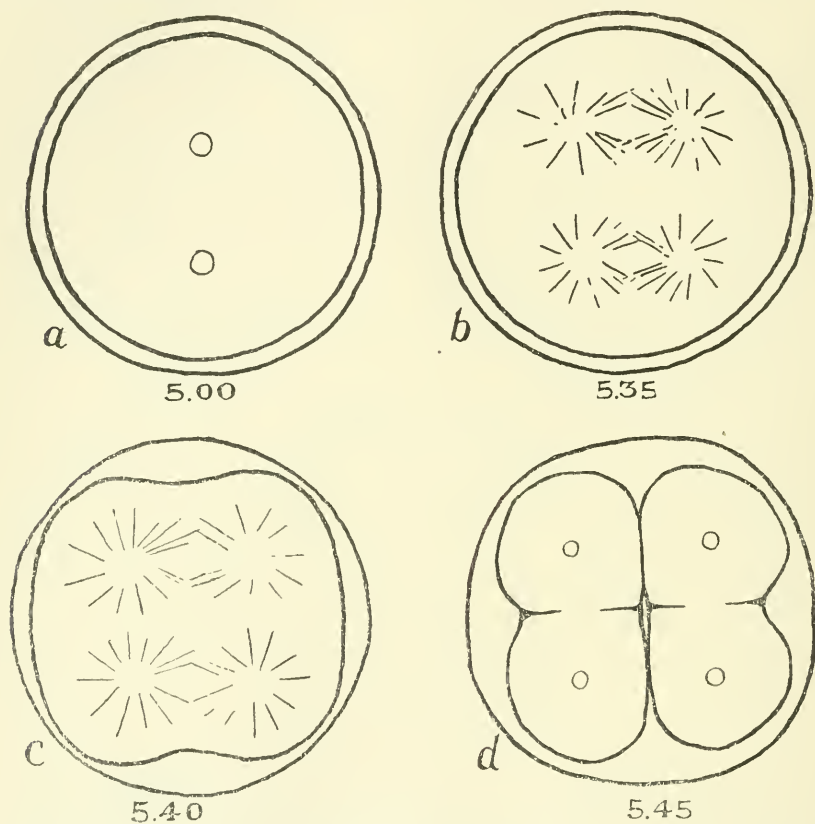


FIG. 8. Development of an *Asterias* ovum manipulated with a needle so as to suppress the first cleavage furrow. *a*, 5.00 p.m., disappearance of the amphister and obliteration of the cleavage furrow. *b*, 5.35 p.m., appearance of two amphisters. *c*, 5.40 p.m., change in shape of the ovum with appearance of second cleavage furrow ahead of the first. *d*, 5.45 p.m., ovum cleaving into four blastomeres. (The ovum developed into a normal embryo.)

Experiment 3.—(Fig. 8.) In this case the first segmentation furrow was prevented from forming by tearing at the equator whenever it made its appearance. The progressive changes within the egg were

undisturbed. As soon as the amphiaster disappeared there was no longer a tendency for the furrow to form (Fig. 8 *a*). The two nuclei now lay in a fluid cytoplasm. Within half an hour after the suppression of the first segmentation furrow, an amphiaster developed about each nucleus preparatory to the next division. The two amphiasters lay side by side but remained distinct from one another, no connecting radiations being formed (Fig. 8 *b*). The formation of the two amphiasters resulted in the transformation of the egg substance into four semirigid bodies, the four asters. Cleavage furrows now extended into the fluid regions between the asters and divided the egg almost simultaneously into four blastomeres. The furrow corresponding to the second cleavage started to form and cut through the egg about a minute ahead of that of the first (Fig. 8 *c* and *d*).

This experiment may throw light on the nature of the segmentation in ova in which several nuclear divisions follow one another with no outward manifestation of the segmentation of the egg. After a certain period the ovum breaks up simultaneously into as many blastomeres as there are nuclei. This is the normal method in certain *Actinozoa* and can be artificially produced in many eggs by exposing them to various reagents, notably hypertonic solutions.^{6,7}

The solidification associated with the aster formation divides the egg cytoplasm into a number of bodies each surrounding a nucleus. Between successive divisions the cytoplasm reverts to a more fluid state but its viscid nature may suffice in preventing the merging of neighboring areas. After a varying number of nuclear divisions with accompanying solidification periods furrows suddenly appear between these bodies and the ovum tends to break up at once into separate blastomeres. A differentiation of this type may possibly have taken

⁶ Loeb, J., Investigations in physiological morphology. III. Experiments on cleavage, *J. Morph.*, 1892-93, vii, 253. Norman, W. W., Segmentation of the nucleus without segmentation of the protoplasm, *Arch. Entwcklnsmechn. Organ.*, 1896, iii, 106. Wilson, E. B., Experimental studies in cytology. I, *ibid.*, 1901, xii, 529. Lillie, R. S., Fusion of blastomeres and nuclear division without cell division in solutions of non-electrolytes, *Biol. Bull.*, 1902-03, iv, 164.

⁷ Wilson, E. B., Experimental studies in cytology, II and III, *Arch. Entwcklnsmechn. Organ.*, 1902, xiii, 353.

place in the unsegmented *Chaetopterus* embryos experimentally produced by Lillie.⁸

Experiment 4.—(Fig. 9.) Fig. 9 *a* to *e* depicts the case of an egg with the cleavage furrow just beginning in which the diagonal cut was incomplete so that the two pieces remained connected at one end of the cut. The original furrow persisted for a time during which it deepened considerably. 30 minutes after the cut had been made

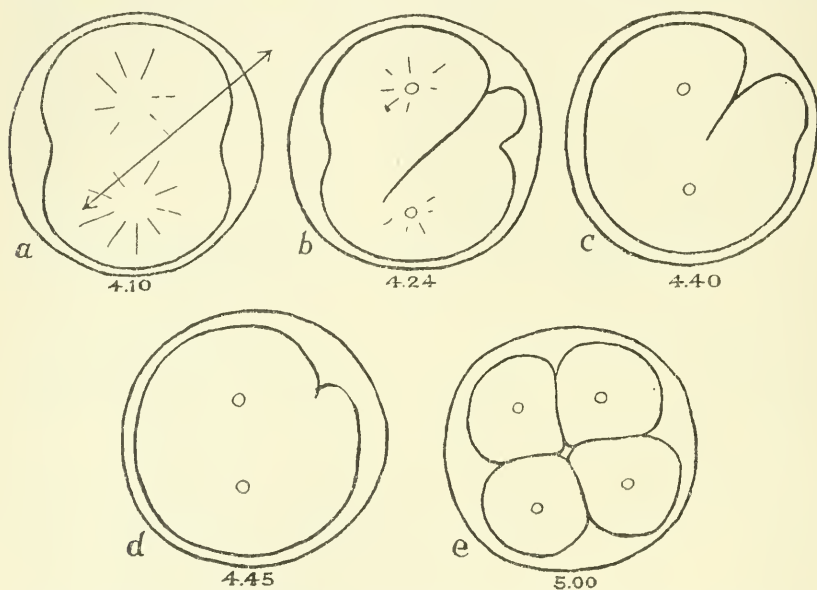


FIG. 9. Effect on an *Asterias* ovum of a deep cut which did not persist. *a*, operation performed at 4.10 p.m. *b*, *c*, and *d* show the egg respectively at 4.24, 4.40, and 4.45 p.m. Both the cut and the cleavage furrow disappear together with a reversal of the ovum from a semisolid to a more fluid state. *e*, 5.00 p.m., the ovum has divided into four normal blastomeres. (The ovum developed into a normal embryo.)

no sign of astral radiations were present and both the original segmentation furrow and the cut produced by the needle were being obliterated (Fig. 9 *c* and *d*). At 5 p.m. the egg had divided into four apparently normal blastomeres (Fig. 9 *e*) and was only slightly

⁸ Lillie, F. R., Observations and experiments concerning the elementary phenomena of embryonic development in *Chaetopterus*, *J. Exp. Zool.*, 1906, iii, 153.

behind the normal controls. By the next morning it had developed into a swimming blastula not to be distinguished from the normal controls.

The obliteration of the cut and of the furrow is consequent to a reversal of the egg cytoplasm from a semirigid to a more fluid state. The film projecting into the egg gradually merges into the liquid

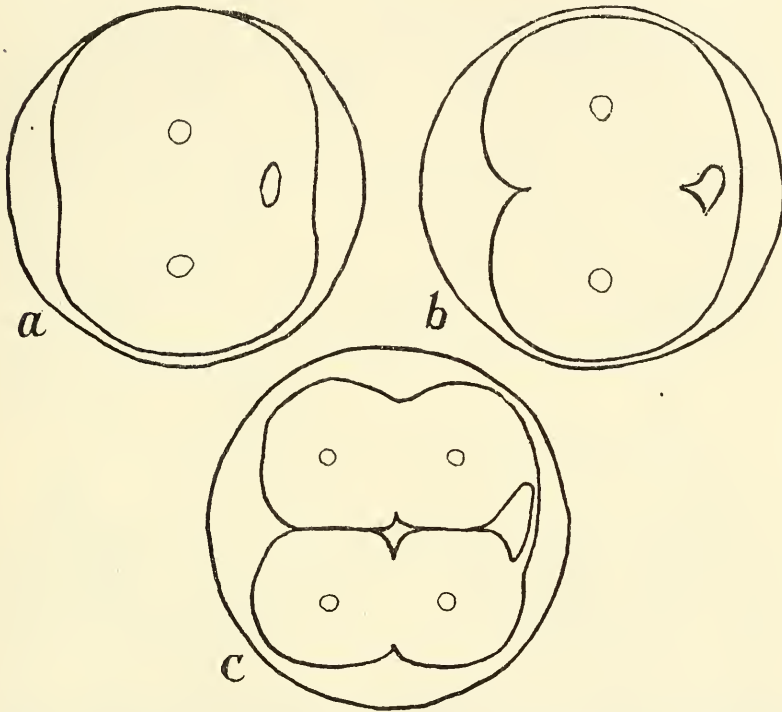


FIG. 10. Successive stages of an *Asterias* ovum showing persistence of a puncture made below the first cleavage furrow as it is beginning to form.

cytoplasm surrounding it and surface tension forces finally overcome the deformation of the egg. The egg now proceeded to divide into four blastomeres as in Experiment 3.

Experiment 5.—(Fig. 10.) This experiment demonstrates a peculiar property of the equatorial region during the formation of the cleavage furrow. A tear was made through the egg below the segmentation furrow (Fig. 10 *a*). The hole produced by the tear

remained open. The cleavage furrow continued its course beneath the hole leaving an outer margin as a bridge of protoplasm which connects the two blastomeres (Fig. 10 *b, c*). After several divisions of the egg the bridge thinned down in its middle until it broke through and the resulting strands were gradually drawn into the blastomeres from which they had projected.

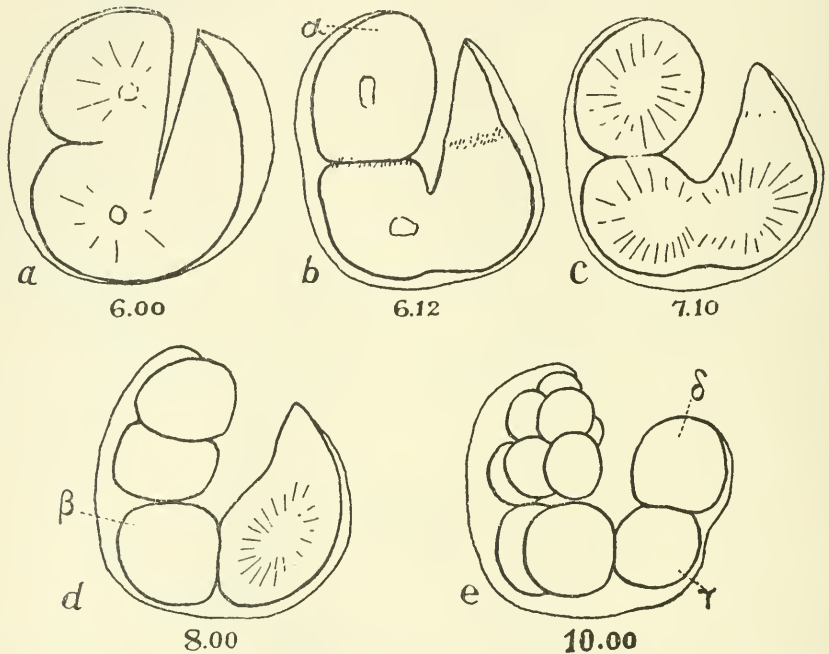


FIG. 11. Effect on an *Arbacia* ovum of a deep cut which persisted. For description of the results see text. Pigment granules collect in plane of original furrow.

Experiment 6.—(Fig. 11.) This experiment was performed on an *Arbacia* egg. An incomplete cut was made almost perpendicular to the cleavage furrow but to one side of the daughter nuclei. The furrow on the side away from the daughter nuclei became obliterated (Fig. 11 *a*). On the other side it continued its original course resulting in the pinching off of the nucleated Blastomere α (Fig. 11 *b*). The nucleus in the remainder of the egg shifted its position only slightly and

the amphiaster (Fig. 11 *c*), forming about it, resulted in a second unequal cleavage with the formation of Blastomere β (Fig. 11 *d*). The projecting piece of the egg above the obliterated furrow remained quiescent during these divisions and not until after the third unequal cleavage resulting in the formation of Blastomere γ in Fig. 11 *e*, did it become incorporated in Blastomere δ .

In this experiment the cut was probably made in the egg when the process for the first cleavage was too far advanced for the egg to retrace its course. The gash was therefore not obliterated and a very peculiar condition resulted in a succession of advances of the cleavage process about the gash. Blastomere α , being the earliest formed, segmented ahead of its fellows (Fig. 11 *d*). Blastomere β came next (Fig. 11 *e*). Unfortunately before Blastomeres γ and δ divided the egg died.

It is significant that Blastomere δ is larger than γ as evidently the former finally incorporated the hitherto inactive part of the egg that lay above that part of the original first cleavage furrow which lay on the right side of the gash (Fig. 11 *b*).

III. Concerning the Mechanism of Cell Division.

The changes in shape that an echinoderm egg undergoes during cleavage can be in part understood on the assumption that the astral formation is a solidifying process. It has long been known that at the time of cleavage the eggs of echinoderms, many worms, mammals, etc., become elongated,⁹ the cleavage furrow forming in a plane at right angles to the long axis of the egg. As the furrow deepens, each resulting blastomere tends to assume the shape of a sphere (Fig. 12 *a*).

Nobody, however, has thus far been able to explain the cause of this elongation. The observations recorded in this paper may explain this phenomenon. The two spheres of solidification grow at the expense of all but possibly a small peripheral part of the fluid egg substance. The combined diameters of the two fully formed semisolid spheres are greater than the original diameter of the egg,

⁹Hertwig, O., Beiträge zur Kenntniss der Bildung, Befruchtung und Theilung des thierischen Eies, *Morph. Jahrb.*, 1876, i, 347. Gurwitsch, A., *Morphologie und Biologie der Zelle*, Jena, 1904.

and hence the egg must elongate. After elongation the surface of the egg seems to tear in the plane separating the two semisolid spheres. The periphery of the two asters of the amphiasier stage never becomes so firm as their interior. This may account for the observation of von Erlanger,¹⁰ confirmed by Spek,¹¹ who described peripheral currents in the rapidly dividing nematode egg. In this egg peripheral currents flow from the two poles toward the equator and from there inward to the center of the egg. Spek suggests that such currents exist in all dividing eggs, and that they are easily visible in the nematode egg because of the great rapidity with which it segments. Conklin¹² described an inward flow of granules at the equator of the dividing *Crepid-*

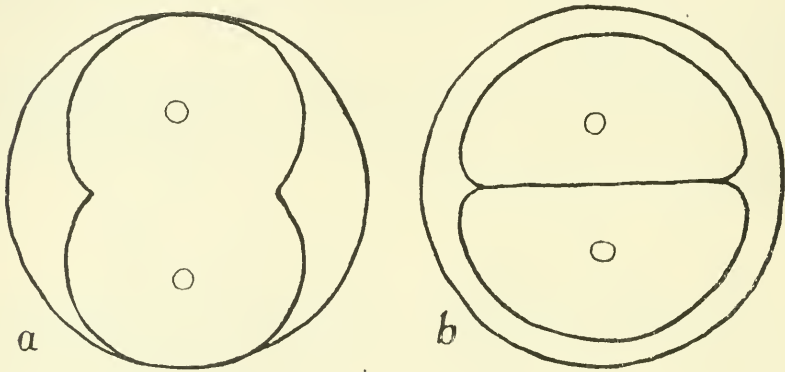


FIG. 12. Change in shape of an *Asterias* ovum (a) before and (b) after completion of the first cleavage furrow.

ula egg, and I³ have observed a similar current, although a very slow one, in the sand-dollar egg.

Immediately after cleavage both of the two blastomeres are more or less spherical; but later, when they become more fluid, they are pressed against each other so as to be flattened at the plane of contact.

¹⁰ von Erlanger, R., Beobachtungen über die Befruchtung und ersten Teilungen an den lebenden Eiern kleiner Nematoden, *Biol. Centr.*, 1897, xvii, 152, 339.

¹¹ Spek, J., Oberflächenspannungsdifferenzen als eine Ursache der Zellteilung, *Arch. Entwicklungsmechn. Organ.*, 1918, xlv, 5.

¹² Conklin, E. G., Protoplasmic movement as a factor of differentiation, *Marine Biol. Lab., Biol. Lect.*, 1899, 69.

Wilson,⁷ in producing binucleate eggs by artificially obliterating the first cleavage furrow, noted that when this was caused by shaking, the resulting binucleate eggs retain the elongated shape (Fig. 13) characteristic of the egg in cleavage. During the ensuing pause (corresponding to the completion of the first cleavage and when

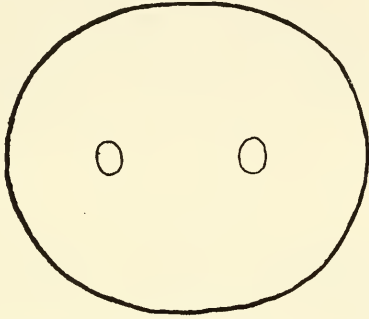


FIG. 13. Copy of Fig. 58 from Wilson⁷ of *Toxopneustes* ovum immediately after shaking which caused obliteration of the first cleavage furrow.

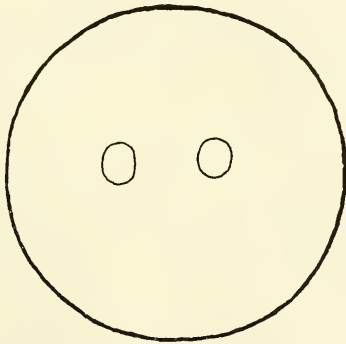


FIG. 14. Copy of Fig. 34 from Wilson⁷ of *Toxopneustes* ovum in which obliteration of the first cleavage furrow was produced by exposure to ether.

the astral radiations fade out preparatory to formation of a new amphiaser system) the egg becomes more nearly spherical. Evidently the shaking does not necessarily produce a reversal of the semisolid astral system to the more fluid state. As soon, however, as this occurs (in the ensuing pause) the egg resumes its spherical shape.

Wilson noted that the suppression of the cleavage furrow can also be produced by placing eggs, during their anaphase stage, in a 2.5 per cent ether solution. The astral radiations disappear and the resulting binucleate egg at once resumes the shape of a sphere (Fig. 14). This phenomenon may be comparable to the experiments illustrated in Figs. 6, 8, and 9 where the obliteration of the astral radiations follows a precocious reversal of the cytoplasm to the more fluid state. The suppression of the furrow in these cases seems to be primarily effected by the change in the physical state of the egg substance which, on reverting to a more fluid state, merges into a single spherical mass.

CONCLUSIONS.

1. The development of the amphiaster is associated with the formation of two semisolid masses within the more fluid egg substance.

2. The elongation of the egg during cleavage is possibly produced as a consequence of the mutual pressure of these two growing semisolid masses.

3. The division of the egg into two blastomeres consists essentially in a growth, within the egg, of two masses of material at the expense of the surrounding cytoplasm. When all the cytoplasm of the egg is incorporated in these two masses cleavage occurs.

4. After a certain period of time the semisolid masses revert to a more fluid state. In the eggs studied this normally occurs after the cleavage furrow has completed the separation of the two blastomeres. The formation of the furrow, however, may be prevented in various ways, upon which the egg reverts to a single spherical semifluid mass containing two nuclei.

5. An egg mutilated during its semisolid state (amphiaster stage) may or may not revert to a more fluid state. If the more solid state is maintained, the cleavage furrow persists and proceeds till cleavage is completed. If the mutilation causes the egg to revert to the more fluid state the furrow becomes obliterated and a new cleavage plane is subsequently adopted.

6. The nuclei of eggs in the semifluid state are able to alter their positions. In semifluid mutilated eggs the nuclei tend to move to positions which may assure symmetry in aster formation and cleavage.

A CHANGE IN THE BAR GENE OF DROSOPHILA INVOLVING FURTHER DECREASE IN FACET NUMBER AND INCREASE IN DOMINANCE.*

By CHARLES ZELENY.

(From the Zoological Laboratory of the University of Illinois, Urbana.)

(Received for publication, July 15, 1919.)

As part of a study of the factors controlling eye facet number in *Drosophila melanogaster*, selection has been carried on with a view to the determination of the germinal changes appearing during the course of selection. One of the most interesting of these changes occurred during the second generation of downward selection in the white bar stock. An exceptionally "low" male of this generation was shown to possess a changed gene with a marked increase in dominance. This individual appeared on November 20, 1917, and had but 19 facets, while the mean of its 62 brothers was 82.6 facets with a range of 41 to 134. With class sizes such that the range of a class is 10 per cent of its mean this represents a departure of -16.80 10 per cent class units from the mean of the males of the unselected bar stock, while the departure of the brothers of the mutant is only -3.44 units. The new stock derived from the third hybrid generation of a mating of the mutant with a sister possesses the characteristics of the original male, and at the present writing (May 10, 1919) has remained without change for nearly 18 months except for the appearance of a few additional mutants.

The name ultra-bar has been given to the new character. Its symbol, B^u , indicates that ultra-bar is a dominant allelomorph of bar. Full-eyed (wild-type) females at 27°C . have an average facet number of 810.6, bar females of the second low selected generation have 61.8 facets, and ultra-bar females 22.0 facets. The corresponding numbers for males are respectively 849.8, 75.6, and 23.0.

*Contribution from the Zoological Laboratory of the University of Illinois, No. 140.

The change in dominance as a result of the mutation is very striking. A cross between a 35.2 facet bar stock of the twenty-fourth generation of the low selection line and an 810.6 facet full-eyed stock gives 399.9 facets in the females, while a cross between the 22.0 facet ultra-bar and the 810.6 facet full gives only 36.5 facets. Ultra-bar thus has a much greater degree of dominance than bar. This is further shown by the cross between the 22.0 facet ultra-bar stock and the 61.8 facet bar stock of the second generation of the low selection line which gives 26.3 facets. Calculated on the basis of 10 per cent class units the ultra-bar has pulled the full-eye down 85 per cent of the distance between the two, while bar has pulled it only 23 per cent. Likewise ultra-bar pulls bar down 85 per cent of the distance between the two, while bar pulls ultra-bar up only 15 per cent.

That the factor for the new character is located in the X chromosome is shown by the reciprocal matings between ultra-bar and bar. Ultra-bar females mated with bar males give ultra-bar males, while the reciprocal matings give bar males.

In order to determine the locus of the new factor the crossing over test was applied. If the new factor is at a different locus from that of bar, crossing over should take place between the two. A mating between ultra-bar and full-eye should then give some bar males in the second hybrid generation, the percentage of such individuals depending upon the distance between the locus of bar and that of ultra-bar. In case, however, the new locus is identical with that of bar there should be no bar males in this generation.

Crosses between ultra-bar females and wild full males give in the second hybrid generation only three males which are not ultra-bar as opposed to 1,238 ultra-bar males. These three males have 88, 51, and 60 facets respectively, all being within the range of bar-eye. On the basis of crossing over this would mean that the locus of the new factor is only 0.24 units from the bar locus on the chromosome map. Unfortunately one of the three males was overetherized. Breeding tests of the other two show a degree of dominance over full which is different from that of bar and they therefore cannot be the result of crossing over. They apparently represent a new mutation.

The most reasonable conclusion to be drawn from these tests is that the locus of ultra-bar is the same as that of bar or that it is

so close to bar as to act as a unit with it for all demonstrable cases. The present case is then an instance of a second mutation in the same germinal material and in the same direction as a previous mutation.

The ultra-bar mutant adds to the series of germinal changes which have been made out in the case of eye-facet number. As previously reported germinal changes have been obtained which involve accessory factors in other than the sex chromosome, some of these producing an effect of large degree and others of small degree. Reverse mutations of the bar stock to full have also been observed. The case described in the present paper is a further step in the same direction as bar. In addition to the further decrease in facet number ultra-bar has a much greater degree of dominance than bar. Furthermore it occurred in the direction of selection, appearing in the low and not in the high selection line.

ADRENALIN IN ANNELIDS.

A CONTRIBUTION TO THE COMPARATIVE STUDY OF THE ORIGIN OF THE SYMPATHETIC AND THE ADRENALIN-SECRETING SYSTEMS AND OF THE VASCULAR MUSCLES WHICH THEY REGULATE.

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(Received for publication, July 16, 1919.)

The intimate physiological relationship between the sympathetic nervous system and the adrenalin-secreting cells of the medulla of the suprarenal body is now thoroughly established. All the actions of the sympathetic system can be imitated by adrenalin, and failure of the adrenalin supply, such as takes place in Addison's disease, causes failure of the proper action of the sympathetic nerves. The morphological relationship of the two types of cell, the sympathetic nerve cell and the adrenalin-secreting cell, are also equally intimate. The adrenalin-secreting cell is always identifiable in mammals by its chrome-staining reaction. Elliott¹ has brought forward strong evidence that the innervation of the medullary cells of the suprarenal body is a direct one by the medullated connector or tract fibers of the splanchnic nerves. Such an innervation corresponds to the white rami communicantes which connect with the sympathetic nerve cells in their various ganglia.

In the higher mammals the sympathetic ganglia do not usually contain any adrenalin-secreting cells, the latter being concentrated almost entirely in the special tissue of the suprarenal medulla; but in the lower forms of vertebrates the two tissues are both widely distributed and intimately connected. In amphibia, for instance, islets of chrome-staining tissues are to be found in every sympathetic ganglion,

¹ Elliott, T. R., *J. Physiol.*, 1913, xlv, 285.

and Smirnow² has shown that such cells are innervated by nerve fibers which are otherwise indistinguishable from those which run to the sympathetic nerve cells lying with them. Further back in the vertebrate kingdom the distribution becomes still more diffuse and the sympathetic nervous system becomes less and less defined and more and more replaced by a diffuse system of chrome-staining cells arranged segmentally throughout the body.

A definite sympathetic nervous system has been long known in the case of the elasmobranch fishes, but it is only comparatively recently that the researches of Giacomini and others have definitely demonstrated its presence in the other groups of fishes. Giacomini finds a definite double sympathetic chain present in the Dipnoi,³ but in the Ganoidei⁴ and Teleostei⁵ he was able to find only an irregular system of nerve cells distributed along the cardinal veins. In the cyclostomes⁶ the representatives of the sympathetic nervous system are still more scanty, being certain nerve cells which are occasionally to be found around the cardinal veins. In all these groups of fishes there is again an intimate relationship between the sympathetic nerve cells and diffusely distributed chrome-staining cells. The presence of adrenalin in the latter cells was demonstrated by Vincent in the elasmobranchs,⁷ and in a previous paper I⁸ gave reasons for believing that the chrome-staining tissue of the cyclostome, *Petromyzon fluviatilis*, also contained adrenalin. An extract of such tissue caused a rise of blood pressure in the cat, which was in every way similar to that caused by a small dose of adrenalin. The conclusion may therefore be drawn that chrome-staining tissue, wherever found among vertebrates, secretes adrenalin, and the presence of a sympathetic nervous system and an adrenalin-secreting system may be considered to have been established throughout the vertebrate kingdom. The two systems are in every animal most intimately connected physio-

² Smirnow, A., *Arch. mikr. Anat.*, 1890, xxxv, 416.

³ Giacomini, E., *Atti Accad. Lincei, Rendiconti*, 1906, xv, series 5, 394.

⁴ Giacomini, E., *Monitore zool. ital.*, 1904, xv, 20.

⁵ Giacomini, E., *Monitore zool. ital.*, 1902, xiii, 183.

⁶ Giacomini, E., *Monitore zool. ital.*, 1902, xiii, 143.

⁷ Vincent, S., *Proc. Roy. Soc. London*, 1897, lxi, 64.

⁸ Gaskell, J. F., *J. Physiol.*, 1912-13, xlv, 59.

logically and anatomically. The work of Kohn⁹ has also shown how closely their evolution in the vertebrate is paralleled by their embryological development in the mammal.

A search for the origin of the two systems must therefore be pursued in the invertebrate kingdom. If it is allowed that the presence of a yellow coloration after fixation with a chrome salt in certain cells, which have been called chromaffin cells, occurs among vertebrates only in cells which contain adrenalin, the presence of such a reaction in cells of invertebrates is presumptive evidence that the latter are also adrenalin-secreting cells. The first discovery of chromaffin cells in the invertebrate was made by Poll and Sommer¹⁰ who described the occurrence of the reaction in certain cells of the central nervous system of the leech, *Hirudo medicinalis*. Poll¹¹ has since extended this observation by finding similar cells in certain other annelids. The only other observation is that of Roaf and Nierenstein¹² who extracted an adrenalin-like body from certain tissues lying in the walls of the branchial chamber of the mollusk, *Purpura lapillus*. Subsequently Roaf¹³ located this secretion to certain cells which gave a chromaffin reaction. As the Mollusca are not held to be on the direct line of vertebrate descent attention has been confined to the annelid kingdom.

A representative selection of annelids was examined at the Zoological Station of Naples, and a detailed description of the results has been given in my paper published in 1914.¹⁴ Among the Hirudineæ chromaffin cells have been found in the ganglia of the central nervous system in all species investigated. Seventeen different animals of the polychæte group were examined but in fifteen of these no trace of chromaffin cells could be found; in the remaining two, *Aphrodite aculeata* and *Eunice gigantea*, small chromaffin cells were present. *Lumbricus herculeus*, the only oligochæte investigated, also possessed

⁹ Kohn, A., *Arch. mikr. Anat.*, 1903, lxii, 263.

¹⁰ Poll, H., and Sommer, A., *Arch. physiol.*, 1903, 549.

¹¹ Poll, H., in, Hertwig, O., *Handbuch die vergleichende und experimentelle Entwicklungslehre der Wirbeltiere*, Jena, 1906, iii, pt. 1, 603.

¹² Roaf, H. E., and Nierenstein, M., *J. Physiol.*, 1907, xxxvi, p. v.

¹³ Roaf, H. E., *Quart. J. Exp. Physiol.*, 1911, iv, 89.

¹⁴ Gaskell, J. F., *Phil. Trans. Roy. Soc. London, Series B*, 1914, ccv, 153.

them. Wherever they were found these chromaffin cells were always constant in number and similar in position in the ganglion, six being always present arranged in three groups of two each, a ventral and two lateral groups. The two cells of the ventral group were usually the largest. The size of the cells varied greatly in the different animals, being small in the two polychætes and reaching the largest size in *Hirudo medicinalis*, which was therefore selected for further investigation.

In this animal the two ventral cells are of very large size; they have been called colossal or giant cells by Retzius¹⁵ and others. The lateral group on each side consists of two smaller cells which lie respectively just posterior to the anterior and posterior lateral nerves; their positions are indicated in Fig. 1. These six cells have all the appearance of nerve cells, possessing processes which run out in the lateral nerves and staining similarly to the other nerve cells of the ganglion. It was found that the six cells could be very clearly demonstrated by staining the freshly excised ganglion with methylene blue, and then irrigating the preparation mounted in water under a cover-slip with a dilute bichromate solution; all the nerve cells became bleached by this process with the exception of the six chromaffin cells which retained the blue stain. The probable explanation of this reaction is that the bleaching action of the chrome salt is prevented in the chromaffin cells by its combination with the chrome-staining substance, thus protecting the methylene blue stain. The nerve cells of the ganglion of the leech are divided into groups, shut off from one another by septa, whose arrangement is shown in Fig. 1. The individual nerve cells are unipolar and hang free in their particular compartment lying in a clear nutritive fluid; their relative positions are therefore liable to alterations in the compartment, for instance the two giant cells can be moved about freely by pressure on the cover-slip in a suitable preparation. The nerve cord itself is suspended in a blood space known as the ventral sinus, the thin sheath of the ganglion intervening only between the compartments in which the nerve cells lie and the surrounding blood. It is quite possible that an interchange takes place between the fluid in which the nerve cells are suspended and the blood

¹⁵ Retzius, G., *Biol. Untersuch.*, 1891, ii, 13.

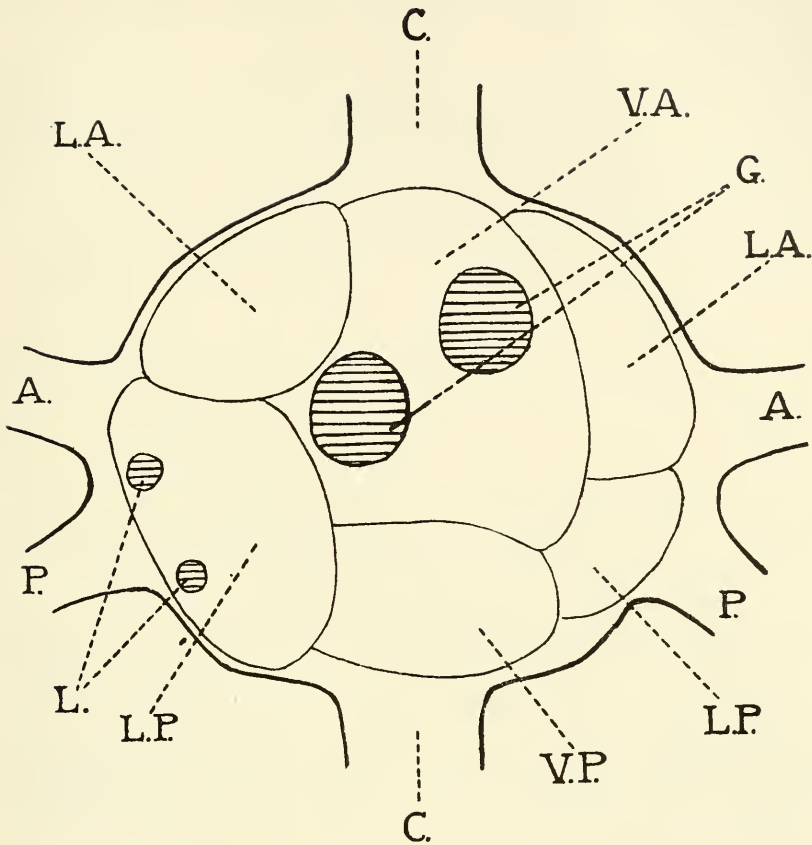


FIG. 1. Diagram of a ganglion of the leech showing the compartments in which the nerve cells are grouped and the position of the chromaffin cells in them. The limits of the various cell groups are shown, with regard to the ventral side of the ganglion on the right and center, with regard to the dorsal side on the left. *G.*, the two giant cells lying in the ventral anterior group. *L.*, the lateral cells lying in the dorsal portion of the posterior lateral groups. *V.A.*, the ventral anterior group. *V.P.*, the ventral posterior group. *L.A.*, the lateral anterior group. *L.P.*, the lateral posterior group. *C.*, the connectives. *A.*, the anterior nerves. *P.*, the posterior nerves.

of the sinus, and that any secretion, such as that of adrenalin in the chromaffin cells, could thus reach the circulation of the segment in which the ganglion lies.

An attempt was made to obtain an extract of the central ganglia in order to see whether any physiological reaction could be obtained similar to that of adrenalin. The nerve cords of a number of leeches were removed under a dissecting microscope by opening up the ventral sinus and cutting through the lateral nerves as near to the ganglia as possible. Each ganglion was then cut away from its connectives and placed immediately in a watch-glass which was kept in a desiccator. About 400 ganglia were thus collected. As the amount of material when dried was extremely small, the physiological test decided upon was the inhibition of the virgin uterus in the cat. I was fortunate in obtaining the assistance of Dr. H. H. Dale in carrying out the test. The uterus was suspended in Ringer's solution and a sufficient amount of histamine was added to the bath to give it a strength of 1 in 3 millions, in order to produce tone and rhythm. The result of the experiment is shown in Fig. 2. An extract of the dried ganglia was made by grinding them up with sand in 2 cc. of Ringer's solution. The bath in which the uterus was suspended contained 50 cc. of Ringer. At *A* the 2 cc. of extract were added to this bath, with the result that a distinct lowering of tone and diminution of rhythm took place. Similar experiments with extremely dilute solutions of adrenalin gave a much stronger inhibition when

$\frac{1}{2,000}$ mg. was added to the 50 cc. bath, but a smaller inhibition when

$\frac{1}{20,000}$ mg. was added; the extract therefore contained an amount of

adrenalin lying between $\frac{1}{5,000}$ and $\frac{1}{10,000}$ mg. This experiment supports the conclusion that the chromaffin reaction of the six nerve cells in the ganglion of the leech is due to the presence of adrenalin in them. It confirms a statement of Biedl that he has been able to obtain the biological tests for adrenalin from these cells. I have, however, been unable to find any detailed description of his experiments.

The structure of these adrenalin-containing cells justifies the conclusion that they are nerve cells, and the question arises whether they innervate some special musculature which is susceptible to the action of adrenalin. That is to say, are they the representatives of the sympathetic cells of the vertebrate as well as the representatives of the adrenalin-secreting cells of the suprarenal medulla? If this is so, in this primitive stage of development, such a nerve cell not only

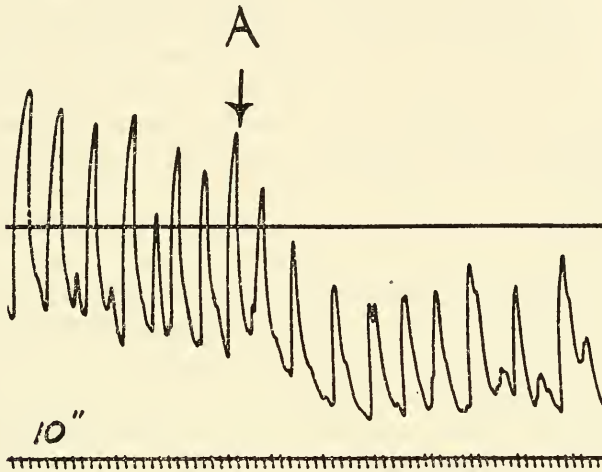


FIG. 2. The action of an extract of leech ganglia upon the virgin uterus of the cat. The isolated uterus was suspended in a bath of 50 cc. of Ringer's solution, to which histamine had been added to produce a strength of 1 in 3 millions; a good tone and rhythm were thus obtained. At *A* the extract of 400 leech ganglia ground up with sand in 2 cc. of Ringer's solution was added to the bath. A diminution in both tone and rhythm was produced. Time markings at intervals of 10 seconds.

regulates its peripheral musculature by direct nervous action but also provides the internal secretion which is necessary for the proper action of its nervous impulses. Later developments have caused a differentiation from this primitive state, so that two separate types of cell have arisen, one of which has become the adrenalin-secreting cell, the other the sympathetic nerve cell. Kohn⁹ considers that in the early mammalian embryo the two types of cell arise from a common mother cell, and may develop into either chromaffin cells or

sympathetic nerve cells. He has therefore called the chromaffin system the paraganglion system, in order to point out its intimate relationship to the ganglia of the sympathetic chains. There is thus a close parallel between the condition in *Hirudo* and the early embryological development of these tissues in the mammal. The connections of the two types of cell with the central nervous system in the vertebrate, which have already been referred to, also support the theory that they have been derived from a common ancestral cell, for both are supplied by medullated connector or tract fibers which run out from the central nervous system to connect with them.

During the investigation of the nervous systems of the various members of the annelid groups, a vascular system with definite muscular walls was found always to exist when chromaffin nerve cells were present, but to be absent when such nerve cells were also absent. The members of the Hirudineæ investigated all possessed muscular walled vessels, and *Lumbricus* has, as is well known, similar muscular "hearts" which are rhythmically contractile. *Eunice gigantia*, one of the two members of the polychæte group in which chromaffin nerve cells were found, possesses a short portion of vessel at the base of each of the branchiæ, which is suspended on a mesentery and has definite muscular walls. These vessels by their contraction drive blood into the branchiæ to be oxygenated. Contractile muscular walled vessels are therefore present in this animal. The vascular system of *Aphrodite* could not be thoroughly investigated owing to lack of material; the question of the presence of vessels with muscular walls could not therefore be decided. In all the other members of the polychæte group, which possessed any definite vessels at all, no sign of any muscular tissue could be found on their walls. It appears that, wherever vascular muscle is present in the Annelida, chromaffin nerve cells are also to be found in the central nervous system, and that these cells are adrenalin-secreting; on the other hand, if no vascular muscle is present no chromaffin cells exist. These facts support the view that the chromaffin nerve cells innervate the vascular muscles.

Further investigations were made to attempt to discover the nature of the innervation of the vascular muscles in the leech, and also their reaction to adrenalin. The nervous system and vascular system were investigated in detail and the results are described in full in my pre-

vious paper.¹⁴ It was found that a small branch of the anterior nerve could be traced directly to the wall of the lateral vessel in each segment, and that this nerve was formed of processes of cells situated in the central segmental ganglion. A branch of the posterior nerve also appeared to run to the same vessel, though its complete continuity was not established. The vascular muscles are therefore innervated by processes of cells situated in the central ganglion, the arrangement being a segmental one.

The lateral vessels were found to have a contractile rhythm with an average beat of about six to eight contractions per minute, and this rhythm was independent of nervous control, for it continued for many hours after complete section of all the lateral nerves. Rhythmical contraction is, in the vessels of the leech, the property of the vascular muscle itself; that is to say, the beat is myogenic not neurogenic.

The effect of section and stimulation of the lateral nerves upon the contractile rhythm of the lateral vessels was also investigated. The study of the vascular beat was much facilitated by the use of curare, which completely paralyzes the longitudinal and circular muscles but does not affect the vascular muscles. The dose required is large, being about 0.2 cc. of a 1 per cent solution, but the differentiation of the two types of muscle by the action of curare is a complete one. Attempts were made to obtain some mechanical method of recording the vascular beat but nothing sufficiently delicate could be devised. The beats were therefore observed under a dissecting microscope and recorded by a key signal on a revolving drum. As the animal had to be kept in Ringer's solution throughout the experiments, stimulation was brought about by means of Lucas'¹⁶ electrode applied to the main nerve chain, the only nerve left intact being the one it was desired to stimulate. It was found that section of the anterior nerve with the posterior nerve intact always caused definite slowing of the rate of beat, while stimulation of this nerve with the posterior nerve divided caused acceleration. The anterior nerve thus contains accelerator fibers to the vascular muscle. Section of the posterior nerve with the anterior nerve intact caused quickening of the rate, while stimulation of this nerve with the anterior nerve divided in the cura-

¹⁶ Lucas, K., *J. Physiol.*, 1913, xlv, p. xxxii.

rized leech had no effect, but in the decapitated animal caused marked slowing of the rate. The posterior nerve therefore contains fibers which are inhibitor to the vascular muscle. The acceleration effect produced by stimulation of the anterior nerve is abolished by the injection of ergotoxin. The vascular system is thus definitely under the control of the central nervous system, the arrangement being a segmental one, and the length of the vessel lying in any segment being controlled by cells in the ganglion of that segment. The accelerator fibers run in the anterior nerve, and their action is abolished by ergotoxin in the same way as is the case with the mammalian sympathetic system. These accelerator fibers have been definitely traced to the muscle and are very probably the processes of the chromaffin nerve cells, which therefore control the vascular muscle in the leech in a similar way to its control in the vertebrate by the sympathetic system. The inhibitor fibers run in the posterior nerve and are also processes of cells in the central ganglion. Their action is abolished by curare. They control the vascular muscle in a manner strictly comparable to the control of the vertebrate heart by the vagus nerve.

The nerve supply of the vascular muscles is therefore a double one which is strictly comparable to the double supply of the vertebrate heart by the sympathetic and vagus systems. The power of rhythmical contraction is an intrinsic property of the vascular muscle itself, to which the two nerves act as regulators. In the primitive form found in annelids, the heart beat is therefore myogenic in origin, but is regulated by the control of the nervous system.

The action of adrenalin was tried both on the longitudinal and circular voluntary muscles and on the vascular muscles. Experiments on the voluntary muscles of the leech were difficult, and were supplemented by similar experiments on *Lumbricus*. No effect could be obtained in these muscles in either animal by the application of adrenalin if it was applied in neutral solution; with the usual acid solution in the form of hydrochloride the acidity of the solution was always sufficient to cause a contraction. Adrenalin borate was found to be a form suitable for the purpose. It was applied directly to a lateral vessel of the leech, after exposing it under Ringer's solution, by injection into the loose tissue lying around the vessel. The effect of the injection of one drop of a solution in Ringer of adrenalin borate

of a strength of 1 in 10,000 is shown in Fig. 3. A beat of an average interval of 14.5 seconds was accelerated to a beat of 9.4 seconds. Later this segment stopped in systole, and the neighboring segments increased their rate of beat. Adrenalin therefore causes a marked acceleration and, if in sufficient strength, it further causes complete contraction which abolishes rhythm altogether. The vascular muscle is sensitive to the action of adrenalin while the voluntary muscle is not; and the action on the vascular muscle is strictly comparable to that on the vertebrate heart.

In order to carry still further the close physiological relationship which had become apparent between the muscles of the vessels of the leech and that of the vertebrate heart, the actions of atropine and muscarine were also tried. Atropine was found to cause an accele-

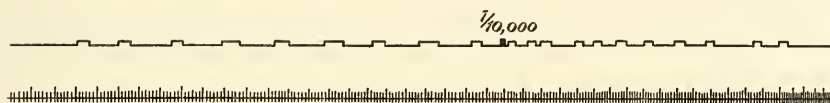


FIG. 3. The action of adrenalin borate on a lateral vessel of the curarized leech. At the signal mark one drop of a solution of adrenalin borate of a strength of 1 in 10,000 was injected into the tissues around the vessel. A beat with an average interval of 14.5 seconds was accelerated to a beat with an average interval of 9.4 seconds. Time marking in seconds.

ration which gave the fastest rhythm ever observed, the average interval between beats dropping to 4.2 seconds, a rate of over fourteen beats per minute. The efficiency of the beat also became maximal. Muscarine in strong solution caused complete cessation of beat in diastole; in dilute solution it caused weakening and slowing of the beat. The complete cessation of the beat caused by muscarine can be removed by a subsequent injection of atropine, if the atropine is injected soon after the beat has ceased. The vessel gradually resumes a rhythmical contraction. If, however, the muscarine has been allowed to act for some time, atropine has no longer the power to recover the beat. These actions of atropine and muscarine are strictly comparable to their actions on the vertebrate heart.

The leech *Hirudo medicinalis* thus possesses a very definite type of vascular muscle clothing certain of its vessels which is rhythmically

contractile and has physiological actions in every way comparable to those of the vertebrate heart. It is controlled by similar nerves and reacts to the drugs adrenalin, atropine, muscarine, and curare in an identical way. The two main vessels, which lie in the extreme lateral position in the body just under the longitudinal muscle layers, are also similar in function. Their chief purpose is to drive blood into an extensive capillary network which lies in the skin and is respiratory in function. The condition in *Eunice gigantea* is probably a primitive form of that in the leech. The segmentally arranged musculature which surrounds a short portion of the vessel lying at the base of each branchia has become more diffusely spread and has fused to form one continuous contractile vessel on each side. In *Eunice* again the function of these "hearts" is entirely branchial. The growing around of the lateral folds of the invertebrate to form the ventral surface of the vertebrate, which is hypothecated in the theory of the origin of vertebrates brought forward by W. H. Gaskell,¹⁷ would carry with them the two lateral vessels. They would thus become mid-ventral and would lie in the position of the two vessels from which embryologically the vertebrate heart is formed. The physiological function of such a heart would be always branchial from its earliest origin in the annelid kingdom.

The formation of a specialized vascular muscle immediately demanded the formation of special nervous and chemical regulators to this muscle; we therefore find a segmental nervous control already established in annelids for the vascular muscle of each segment, and a specialized cell which secretes the necessary internal secretion; namely, adrenalin. In this primitive condition the secretion of adrenalin is a function of nerve cells, constant in number, which are situated in the segmental ganglion and are also in all probability the nervous regulators of the vascular muscle. These cells therefore represent the common ancestors of the sympathetic and of the adrenalin-secreting systems of the mammal. In the course of evolution the two functions have become separated, and two distinct types of cell have arisen, one of which is purely secretory and the other purely nervous. In the earliest vertebrates the secretory system is chiefly

¹⁷ Gaskell, W. H., *The origin of vertebrates*, London, 1908.

in evidence, but it is here most intimately connected with the ganglia and trunks of the posterior nerves, an arrangement strictly comparable with Ōnodi's¹⁸ description of the early development of the mammalian sympathetic system. The evolution in the vertebrate kingdom of the two now separate systems takes the form of a steadily increasing development of the nervous or sympathetic type of cell and a relative diminution and concentration of the secretory type. The final condition reached in the mammal is a widely distributed complex sympathetic nervous system, with a complete concentration of the secretory system into the medullary tissue of the suprarenal capsules.

The emigration from the central nervous system of the cells which secrete adrenalin took place at the same time as that of the nerve cells of the sympathetic system; their close association and similarity of nervous control is still clearly seen in animals as high in the vertebrate scale as the amphibia, where chromaffin cells are incorporated in every sympathetic ganglion.

CONCLUSIONS.

1. The sympathetic nervous system and the adjuvant adrenalin-secreting system are found in their earliest form in the annelid kingdom, and consist of cells situated in the central nervous system which are the common ancestors of both, and which are both secretory and nervous in function.

2. These cells are developed in the annelid kingdom parallel with the development of a contractile vascular system, which possesses muscles comparable in physiological actions with the muscle of the vertebrate heart.

3. This vascular muscle is regulated by the processes of the common ancestral cells as well as by their secretory activity.

4. In the primitive form contractile rhythm is an intrinsic property of cardiac muscle; its nerve supply regulates the rhythm, it does not initiate it. The beat is therefore myogenic, not neurogenic.

5. The contractile vascular system of annelids is mainly branchial in function. The vertebrate heart has been derived from it by the growing around of the lateral body folds to form a new ventral surface.

¹⁸ Ōnodi, A. D., *Arch. mikr. Anat.*, 1885-86, xxvi, 553.

ELECTRIFICATION OF WATER AND OSMOTIC PRESSURE.

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(Received for publication, July 21, 1919.)

I. INTRODUCTION.

When a watery solution is separated from pure water by a strictly semipermeable membrane we call osmotic pressure the additional pressure upon the solution which is required to cause the migration of as many molecules of water from the side of the solution to the side of the pure solvent as migrate simultaneously from pure water to the solution. Van't Hoff's theory demands that this pressure should increase with the number of molecules in the solvent and that it should be equal to the gas pressure of the solute in the volume of the solution. The actual measurements of osmotic pressure of solutions of cane sugar made by Morse¹ and his collaborators as well as by the Earl of Berkeley² and his fellow workers show a rather close approximation to van't Hoff's theory.

When the solution is separated by a strictly semipermeable membrane, the difference in the rate of diffusion of water in opposite directions must be the greatest at the beginning of the experiment, and the difference must diminish steadily during the experiment as a consequence of the increase of hydrostatic pressure on the side of the solution. The value of the osmotic pressure of a solution as defined above must therefore vary with the difference in the rate of the diffusion of water molecules in the two opposite directions at the beginning of the experiment. When we double the concentration of the solute we also double the initial difference in the rate of diffusion of water molecules in opposite directions, and the additional pressure

¹ Morse, H. N., The osmotic pressure of aqueous solutions, *Carnegie Institution of Washington, Publication 198*, 1914.

² Earl of Berkeley, and Hartley, E. G. J., *Proc. Roy. Soc. London, Series A*, 1916, xcii, 477.

which is to be applied to the solution to make the rate of diffusion of water in both directions equal must also be doubled.

This influence of the concentration holds strictly only as long as the solute influences the rate of diffusion of water simply by the number of its molecules (*e.g.* in preventing a number of water molecules from impinging on the solution side of the membrane this number being equal to the number of molecules of solute impinging during the same time). Van't Hoff's law, however, must become inadequate if the molecules of the solute can modify the rate of the diffusion of water by other forces than mere gas pressure; *e.g.*, by electrical forces varying with the nature of the molecule. This is probably true for any solute but in a much smaller degree when the solute is a non-electrolyte than when it is an electrolyte; and in the case of electrolytes it holds in a smaller degree when the electrostatic field around the individual oppositely charged ions is nearly the same (*e.g.* in the case of NaCl) than when it is very different as in the case of $\text{Na}_4\text{Fe}(\text{CN})_6$ or LaCl_3 . This influence of the electrical field surrounding the ions upon the rate of diffusion is due to the electrification of the water molecules.

We use the term electrification of water merely as a short expression of the fact that electrostatic forces cause water to migrate in a definite sense through a membrane. Whether this electrification of water particles is due to a cluster formation of water molecules around an ion as a nucleus, or to some other cause, may for the present remain outside the discussion.

It is the purpose of this paper to show that the electrification of water molecules by ions in solution must in certain cases result in a deviation of the osmotic pressure of a solution from that to be expected on the basis of van't Hoff's theory; and that the sense and relative quantity of deviation can be predicted.

The writer has recently investigated the influence of various ions on the rate of diffusion of water through a collodion membrane separating pure water from a watery solution. Before being used for the experiment the collodion membranes were filled over night with a 1 per cent solution of gelatin and kept in water. The next day the gelatin solution was removed and the collodion flasks were rinsed a considerable number of times with warm water to remove

the remnants of the solution. The initial treatment of the membranes with gelatin apparently modified the collodion permanently since after weeks of daily use these membranes, treated once with gelatin, behaved differently from membranes not treated with gelatin. We shall return to this fact in another publication. An investigation of the influence of electrolytes on the rate of diffusion of water through such collodion membranes previously treated with gelatin has shown that all the phenomena observed can be explained on the basis of the following two rules.

"1. Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion and diminishing inversely with a quantity which we will designate arbitrarily as the "radius" of the ion. The same rule applies to solutions of alkalies.

2. Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte. Solutions of acids obey the same rule."³

These two rules allow us to predict in which sense the *nature* of the electrolyte in solution should modify the osmotic pressure of a solution calculated on the basis of van't Hoff's law. Suppose pure water is separated by a collodion membrane from a watery solution of an electrolyte. When the electrolyte is one of those mentioned in Rule 1, *i.e.* possessing a monovalent or bivalent cation, water is attracted by the solution and diffuses from the side of pure water into the solution, as if the particles of water were positively charged. They should therefore be attracted by the anion and repelled by the cation of the electrolyte and the more so the higher the valency of these ions. Hence water should diffuse more slowly into a solution of $M/192 \text{ CaCl}_2$ than into a solution of $M/128 \text{ NaCl}$, and more slowly into $M/128 \text{ NaCl}$ than into $M/192 \text{ Na}_2\text{SO}_4$, and considerably more slowly into $M/128 \text{ NaCl}$ than into $M/256 \text{ Na}_3\text{PO}_4$ or $M/320 \text{ Na}_4\text{Fe}(\text{CN})_6$. If, however, the water particles are negatively charged everything is reversed, the water diffusing more rapidly into $M/192 \text{ CaCl}_2$ than

³ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 720.

into $M/128$ NaCl, and more rapidly into $M/128$ NaCl than into $M/192$ Na_2SO_4 .

From the theoretical connection between the relative rate of diffusion of water from pure water to the solution through a semipermeable membrane discussed before, it follows that the osmotic pressure of a solution should be modified by the *nature* of the ions it contains in the same sense as the initial rate of diffusion of water is modified. This idea can be put to a test by the choice of electrolytes for which the collodion membrane is strictly semipermeable; *e.g.*, gelatin salts. Gelatin solutions attain in bags of collodion an osmotic pressure which is permanent, provided the hydrogen ion concentration of the solution does not undergo any change during the experiment.

II. Analogies between Solutions of Gelatin and Aluminium Salts.

Gelatin is an amphoteric electrolyte which when the hydrogen ion concentration of its solution exceeds the critical value $2 \times 10^{-5} N$ forms only salts of the type of gelatin chloride, gelatin sulfate, etc., while when its hydrogen ion concentration falls below this value it can form only salts of the form of metal gelatinates; *e.g.*, Na gelatinate, Ca gelatinate, and so on. At the critical hydrogen ion concentration $2 \times 10^{-5} N$ —the isoelectric point for gelatin—it can exist only in the form of pure, *i.e.* non-ionogenic, gelatin.⁴ In this condition gelatin is practically insoluble, practically non-ionized, and is practically incapable of producing any osmotic pressure. Both types of gelatin salts, metal gelatinates as well as gelatin chloride, etc., are very soluble, are strongly ionized, and are capable of producing osmotic pressure. The writer's experiments, which are not yet all published, have shown that for each given hydrogen ion concentration there exists a definite equilibrium between non-ionogenic gelatin, gelatin salt, and free acid. If we have 1 per cent solutions of isoelectric gelatin the ionized or salt portion of the gelatin is practically zero. When we add increasing quantities of an acid, *e.g.* HCl, an increasing portion of the gelatin is transformed into gelatin chloride, while the portion of non-ionogenic gelatin is correspondingly dimin-

⁴ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 39, 237, 363, 483, 559.

ished. The relative proportion of gelatin salt and non-ionogenic gelatin depends therefore upon the hydrogen ion concentration of the solution and increases in a characteristic way with this concentration. This hydrogen ion concentration of the solution is, of course, not identical with the concentration of the acid added to the solution, since part of this acid is in combination with the gelatin forming the gelatin salt. Since practically only that fraction of the 1 per cent gelatin solution which is transformed into a gelatin salt produces an osmotic pressure it is obvious that the osmotic pressure of a 1 per cent solution of gelatin must change in a definite way with the hydrogen ion concentration of the solution. If we use different acids we find that different quantities of acid are required to bring a 1 per cent gelatin solution to the same pH.

When the hydrogen ion concentration is below the critical value 2×10^{-5} (or to use Sørensen's logarithmic symbol, when pH is > 4.7) a part of the non-ionogenic gelatin is transformed into metal gelatinates and this part is the greater the more the hydrogen ion concentration falls below 2×10^{-5} . There is again a definite equilibrium between hydrogen ion concentration, gelatin salt, and non-ionogenic gelatin.

Hence if we wish to compare the osmotic behavior of different gelatin salts we must see that the solutions have not only the same concentration of gelatin but also the same hydrogen ion concentration.⁵

The influence of solutions of gelatin on the electrification of water is the same as that of any other electrolyte and follows the two rules mentioned above. In solutions of metal gelatinates with monovalent or bivalent cations, water migrates through a collodion membrane as if the molecules of water were positively charged; in solutions of gelatin acid salts water migrates as if its particles were negatively charged. The turning point for the sense of migration seems to be near (or identical with) the isoelectric point of gelatin; *i.e.*, pH = 4.7.

⁵ This latter point has been overlooked by the majority of colloid chemists who refuse to admit the chemical nature of the equilibrium between colloid and crystalloid, and who consequently ignore the rôle of the hydrogen ion concentration of the solution. Instead they compare the effect of the addition of equal quantities of different acids, overlooking the fact that they are thus comparing solutions in which the proportion of non-ionogenic gelatin and gelatin salt is different in the case of each acid.

It is perhaps not without interest that the behavior of gelatin is paralleled by the behavior of amphoteric electrolytes of a crystalloid character; *e.g.*, aluminium salts. Thus aluminium chloride exists only when the hydrogen ion concentration exceeds a certain critical value which seems to lie near that of the point of neutrality. When the solution becomes alkaline metal aluminates are formed. Na aluminate as well as AlCl_3 is very soluble. At the isoelectric point neither salt can exist and the insoluble $\text{Al}(\text{OH})_3$ is formed.⁶ The insoluble $\text{Al}(\text{OH})_3$ has no osmotic pressure (or does not attract water) while solutions of both AlCl_3 as well as NaAlO_2 attract water powerfully. In the presence of AlCl_3 water molecules are apparently negatively electrified and in the presence of NaAlO_2 water shows positive electrification. The turning point for the sense of migration of water molecules seems to lie near or at the isoelectric point of aluminium; namely, pH about 7.0. It would be very important if we could measure the permanent osmotic pressure of aluminium salts in collodion bags, but this is impossible since aluminium salts (with the exception of the insoluble $\text{Al}(\text{OH})_3$) diffuse through collodion membranes. It is, however, possible to determine the influence of different aluminium salts upon the rate of diffusion of water through a collodion membrane and it is found that this influence obeys the two rules.

We have mentioned this analogy between an amphoteric crystalloid, AlCl_3 and NaAlO_2 on the one hand, and an amphoteric colloid, gelatin chloride and sodium gelatinate, on the other to show that the fact of diffusibility or non-diffusibility through a collodion membrane does not force us to assume that the gelatin salts form no true solutions.

III. Osmotic Pressure of Different Metal Gelatinates.

Only the metal gelatinates with monovalent and bivalent cations need to be considered since the gelatin salts with trivalent cation seem to be insoluble. Different metal gelatinates were prepared

⁶ The sparingly soluble $\text{Al}(\text{OH})_3$ may be called a colloid since it does not diffuse through parchment paper. Sodium aluminate and AlCl_3 are crystalloids since they diffuse rapidly through such a membrane.

from gelatin rendered isoelectric (in the way described in former papers) by adding LiOH, NaOH, etc., to the gelatin. About 18 cc. of 0.01 N NaOH or $\text{Ca}(\text{OH})_2$ must be contained in 100 cc. of a 1 per cent solution of isoelectric gelatin to obtain a metal gelatinate with a pH of 7.0; *i.e.*, with neutral reaction. We shall call a solution containing 1 gm. of isoelectric gelatin in 100 cc. a 1 per cent gelatin solution notwithstanding the fact that the gelatin is caused to combine with an acid or base.

We will first show that the gelatin salts with monovalent and bivalent cations influence the rate of diffusion of water in the same sense as is done by common crystalloid salts with monovalent and bivalent cations. Since in the presence of such metal gelatinate water diffuses through a collodion membrane as if its particles were positively charged (as ascertained by experiments with a constant current) we should expect that water should diffuse more rapidly into 1 per cent solution of metal gelatinate with monovalent cations, Li, Na, K, NH_4 , than with bivalent cations, Mg, Ca, Sr, Ba, etc. This is indeed the case. We proceeded in the same way as in our previous experiments. Collodion bags,⁷ in the shape of Erlenmeyer flasks with about 50 cc. contents, and all with the same surface, were prepared in as uniform a way as possible. The flasks were closed with a perforated rubber stopper through the opening of which a glass tube (with bore of about 2 mm. diameter) serving as a manometer was pushed. These collodion flasks were filled with water distilled in a tin still and having a pH of about 5.2. The collodion bag was put into a beaker filled with a 1 per cent solution of a metal gelatinate and the pressure head of the water in the manometer tube was at the beginning of the experiment about 120 mm. of H_2O . The fall of the level of the water in the glass tube was measured in definite intervals. In Figs. 1 and 2 the levels are plotted as ordinates over the time elapsed since the beginning of the experiment. The experi-

⁷ The permeability of the collodion bags was tested before each experiment by filling them with M/4 cane sugar and measuring the rate at which water diffused into them. When the water rose in the glass tube with a bore of 2 mm. in diameter to a height of about 110 mm. in 20 minutes the membranes were considered serviceable.

ments were made at a constant temperature of 24° . The two figures show that water diffuses more rapidly into Li and K gelatinate than into Ba and Ca gelatinate. Na and NH_4 gelatinate behaved like Li and K gelatinate. The pH of the gelatin solutions was 8.2 in this experiment.

A second method of testing the influence of different metal gelatinates upon the rate of diffusion consisted in determining that concentration which a cane sugar solution must have in order to balance

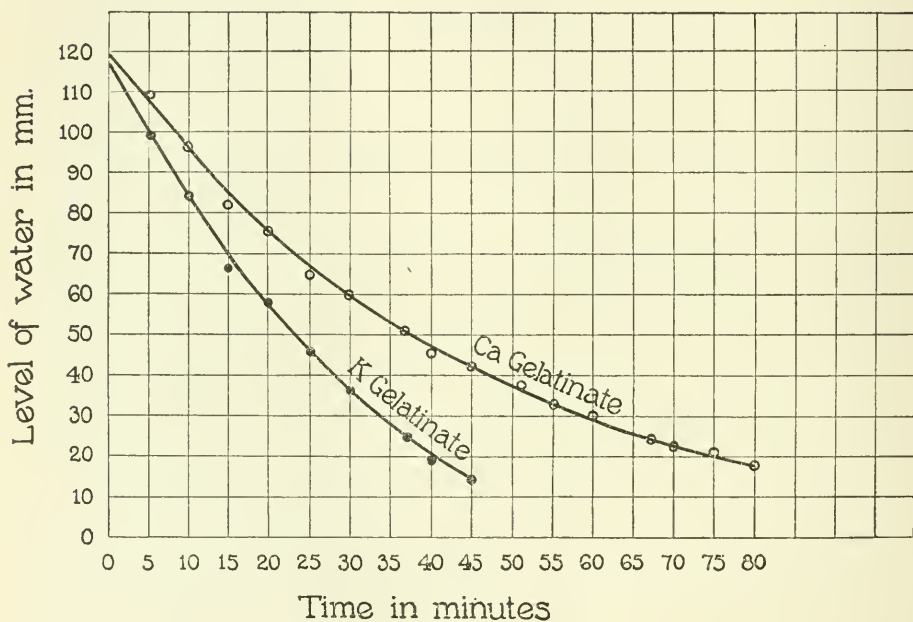


FIG. 1. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solutions of Ca and K gelatinate ($\text{pH} = 8.2$).

the attraction of a 1 per cent solution of metal gelatinate for water. The procedure was as follows. 350 cc. of a 1 per cent solution of a metal gelatinate, *e.g.* Na gelatinate, of $\text{pH} 7.0$ were put into each of a series of beakers and into each beaker was put one of the collodion flasks filled with a different concentration of cane sugar varying from

M/1 to M/64. It was useless to go below a concentration of M/64 of cane sugar since this was about the lowest concentration at which cane sugar influenced the rate of diffusion of water. The level of the cane sugar solution in the manometer tube was about 25 mm. at the beginning of the experiment. When the water diffused more rapidly from gelatin to cane sugar than in the opposite direction, the level in the manometer rose, when the water diffused more rapidly

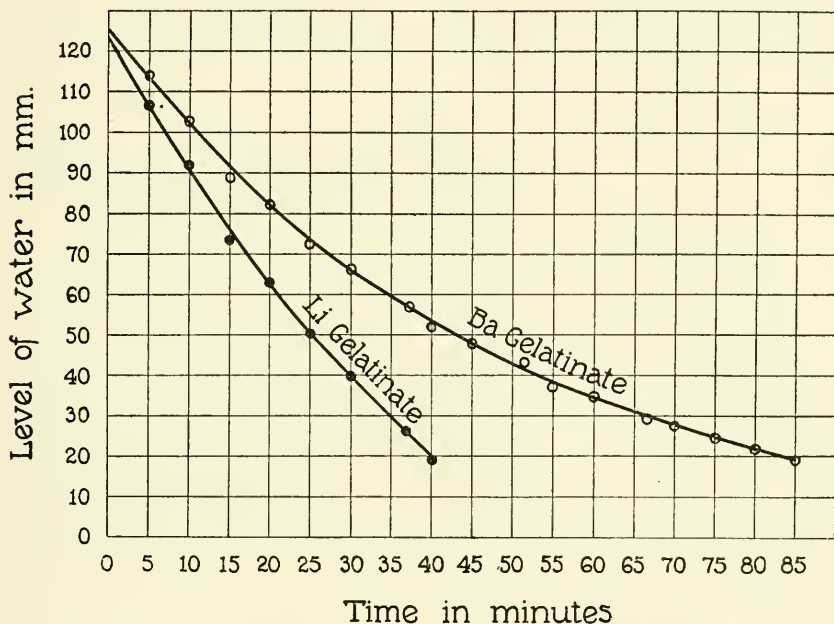


FIG. 2. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solution of Ba and Li gelatinate ($pH = 8.2$).

from cane sugar into gelatin than in the opposite direction the level in the manometer tube fell. Between the two was a concentration where the rate of diffusion in both directions was the same and this concentration of cane sugar we called the balancing concentration of cane sugar. Such experiments are only of value when of short duration on account of the fact that the sugar diffuses out into the gelatin

solution. In Table I the level of the water in the manometer tube after 30 minutes is given for the different 1 per cent gelatin solutions. The plus sign means a rise in the level of water in the cane sugar solution above the original level of 25 mm., the minus sign means a fall of the level of the sugar solution in the manometer tube.

We notice that the concentration which a cane sugar solution must have to balance osmotically 1 per cent solutions of gelatin salts with monovalent cations of a pH of about 7.0 lies between $M/8$ and $3M/32$, while for Ca gelatinate of the same concentration and pH the balancing concentration of cane sugar has a value between $M/32$ and $M/16$,

TABLE I.

Nature of gelatin salt used.	Change of level of liquid in the manometer tube of different concentrations of cane sugar solution when immersed in 1 per cent solutions of different metal gelatinates of pH 7.0, after 30 min.										
	Concentration of cane sugar solution.										
	$M/1$	$3M/4$	$M/2$	$3M/8$	$M/4$	$3M/16$	$M/8$	$3M/32$	$M/16$	$M/32$	$M/64$
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
Li gelatinate.....	358	294	195	109	+60	+24	-4	-11	-16	-21	-16
Na ".....	397	289	251	120	+97	+22	-3	-11	-20	-19	-17
K ".....	379	285	186	102	+62	+22	-3	-7	-15	-18	-22
NH ₄ ".....	390	323	222	127	+96	+35	+4	-4	-16	-19	-20
Ca ".....	397	300	241	172	+99	+69	+33	+16	+2	-2	-18
Ba ".....	376	310	225	145	+97	+77	+36	+29	+12	0	-15

and for Ba gelatinate a slightly lower value. We may, therefore, state that the balancing concentration of cane sugar is roughly over twice as great when the metal gelatinate has a monovalent cation as when it has a bivalent cation. Since water diffuses towards a cane sugar solution with a velocity which in the beginning of the experiment increases in proportion with the concentration of the sugar, we can say that the rate of diffusion of water into 1 per cent solutions of gelatin salts of the type Na gelatinate is between two and three times as great as the rate of diffusion of water into a solution of Ca or Ba gelatinate of the same concentration and pH.

The question now arises; Is there a similar difference between the

permanent osmotic pressure of 1 per cent solution of metal gelatinates when the cation is monovalent and bivalent? We have already published such measurements before we were aware of the fact that the electrification of water might play a rôle in osmotic pressure. In a paper published in this *Journal*⁸ we have shown that when 1 per cent solutions of gelatin salts with monovalent cation—Li, Na, K, and NH_4 gelatinates—are separated by collodion membranes from distilled water they may reach a maximal osmotic pressure of from 300 to 325 mm. of gelatin solution, while the 1 per cent gelatin solutions with bivalent cation—Ca and Ba gelatinates—never reach an osmotic pressure higher than 130 mm.; *i.e.*, a little more than one-third the value of the highest osmotic pressure of 1 per cent solutions of gelatin salts with monovalent cation.

It seemed desirable to get the time curve for the rise in pressure, since such a curve permits a comparison between the influence of the cation on both the velocity of the diffusion of water from water into solution as well as on the pressure when osmotic equilibrium is reached. The experiment was as follows. The gelatin was rendered isoelectric in a way described in a previous paper. To 1 gm. of isoelectric gelatin were added 20 cc. of 0.01 N of an alkali (LiOH, NaOH, etc.) and then enough distilled water to bring the solution of the gelatin (which was melted) to 100 cc. The pH of these solutions in the experiment under discussion turned out to be about 8.8. The solutions were put into the collodion flasks and the latter were put into beakers containing 350 cc. of H_2O , the pH of which was raised at the beginning to about 9.0 by adding 0.2 cc. of 0.01 N NaOH to 350 cc. of H_2O , in order to prevent a rapid lowering of the pH of the gelatin solution through the CO_2 absorbed from the air by the distilled water of the outside solution. The temperature was 24°C . The pH decreased slowly in the beaker and as a consequence also in the gelatin solution, and in 20 hours the pH had fallen to about 7.0 in the gelatin solutions with monovalent cations, while it had fallen less in the gelatin solutions with bivalent cations. When the pH fell, the osmotic pressure also began to diminish on account of the shifting of

⁸ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 483.

the equilibrium between non-ionogenic gelatin and the metal gelatinates formed.⁹

Fig. 3 gives the rise of level of liquid in the manometer during the first 12 hours for a 1 per cent NH_4 gelatinates and a 1 per cent Ba gelatinates solution. Equilibrium was reached after about 6 hours. The slight diminution of osmotic pressure due to the fall of pH on

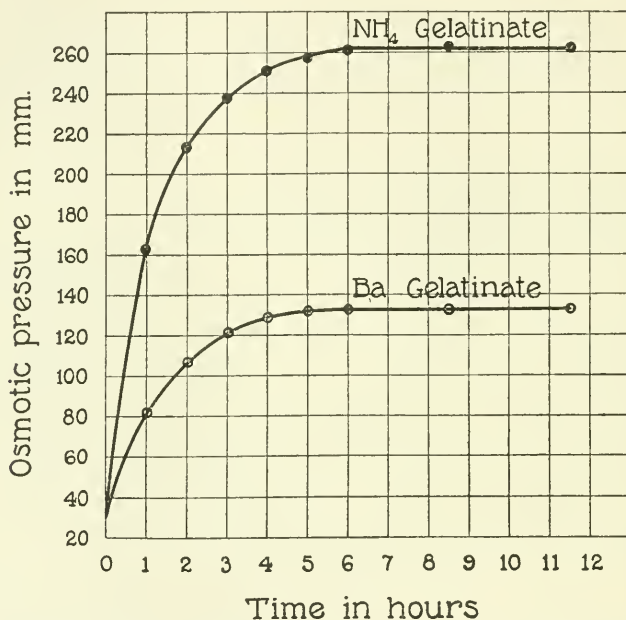


FIG. 3. Curves of rise of osmotic pressure of 1 per cent solutions of NH_4 and Ba gelatinates ($\text{pH} = 8.8$). Pressure in mm. of column of gelatin solution.

account of CO_2 action did not commence until later. The osmotic pressure of the 1 per cent solution of NH_4 gelatinates is in this experi-

⁹ In order to make sure that the osmotic pressure reached is permanent, the experiments with metal gelatinates should be made with the exclusion of CO_2 . Since, however, this source of error does not exist in the case of gelatin acid salts where the permanency of the final pressure reached can be made sure of, and since the results in that case are the same, as we shall presently see, and since the error was less for Ca and Ba gelatinates than for Na gelatinates, we need not dwell upon this point.

ment about twice as high as that of the 1 per cent solution of Ba gelatinate of about the same pH. During the first hour the relative rise of level in the solutions is also approximately as 1:2. Fig. 4 gives the curves for Na gelatinate and Ca gelatinate. The curve for Na gelatinate in Fig. 4 is identical with the curve for NH_4 gelatinate in Fig. 3 and the curve for Ca gelatinate in Fig. 4 is identical with the curve for Ba gelatinate in Fig. 3. The curves for Li and K gelatinate were identical with the curves for NH_4 and Na gelatinate in Figs. 3 and 4. Such results are always obtainable when both the

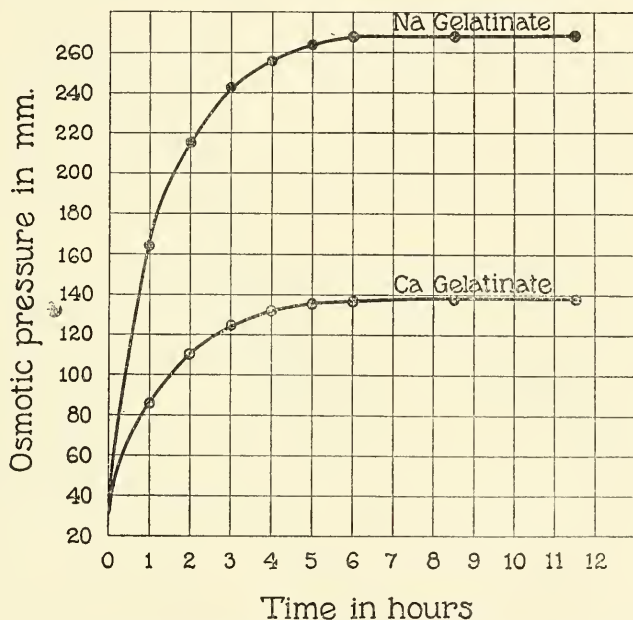


FIG. 4. Curves of osmotic pressure of 1 per cent solutions of Na and Ca gelatinate ($\text{pH} = 8.8$). Pressure in mm. of column of gelatin solution.

concentration and the pH of the solutions are identical. We therefore reach the conclusion that the different metal ions influence the osmotic pressure of gelatin solutions in the same sense as they influence the rate of migration of water into the solution. This supports the idea that the electrification of the water particles plays a rôle in the magnitude of the osmotic pressure as obtained by the use of semipermeable membranes.

IV. *The Gelatin Cation.*

When the hydrogen ion concentration of the gelatin solution is higher than 2×10^{-5} the gelatin can exist only in the form of gelatin chloride, sulfate, citrate, etc. Water is electrified negatively by such gelatin solutions and hence when pure water is separated by a collodion membrane from a 1 per cent solution of gelatin chloride or sulfate, etc., the water should diffuse more rapidly into the gelatin solution when the anion is monovalent than when it is bivalent. We have shown in a former paper¹⁰ that all dibasic and tribasic acids (thus far tried by us), with the exception of sulfuric acid, combine in molecular and not equivalent proportions with gelatin. Thus gelatin forms with phosphoric acid a monogelatin phosphate, with tartaric acid a monogelatin tartrate; only with sulfuric acid does it form a digelatin sulfate. In the case of gelatin oxalate it is possible that we have a mixture of both the monogelatin and the digelatin oxalate, the former prevailing.

This fact is of great importance for our problem. In the case of monogelatin phosphate the anion is not trivalent PO_4 , but essentially the monovalent anion H_2PO_4 . The same is true for monogelatin citrate and in the case of monogelatin oxalate the anion is not a bivalent oxalate ion but a monovalent oxalate ion with one hydrogen attached. Only in the case of gelatin sulfate is the anion divalent. We have shown in our preceding paper that in the case of NaH_2PO_4 the attraction of the salt for water is of the order of magnitude of a salt with monovalent anion like NaCl , showing that the presence of the two H ions weakens the influence of the trivalent PO_4 ions considerably. We therefore should expect that in the case of salts like monogelatin citrate, monogelatin phosphate, monogelatin oxalate, and monogelatin tartrate the influence of anion on the rate of the diffusion of water should be of the same order of magnitude as in the case of gelatin chloride or nitrate; while in the case of digelatin sulfate the SO_4 ion should act as a divalent anion producing a stronger repelling effect on the negatively charged water than is done by gelatin chloride. This turns out as expected (Fig. 5).

¹⁰ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 559.

When we fill the collodion bags as described with distilled water and put the bags into 1 per cent solutions of different gelatin acid salts of the same pH, giving the water inside the bag an initial pressure head of about 125 mm. of H_2O , the water will diffuse out more slowly against the gelatin sulfate than against any of the solutions of the other gelatin salts. This is demonstrated by Fig. 5. The abscissæ are the time in minutes, the ordinates the level of water in

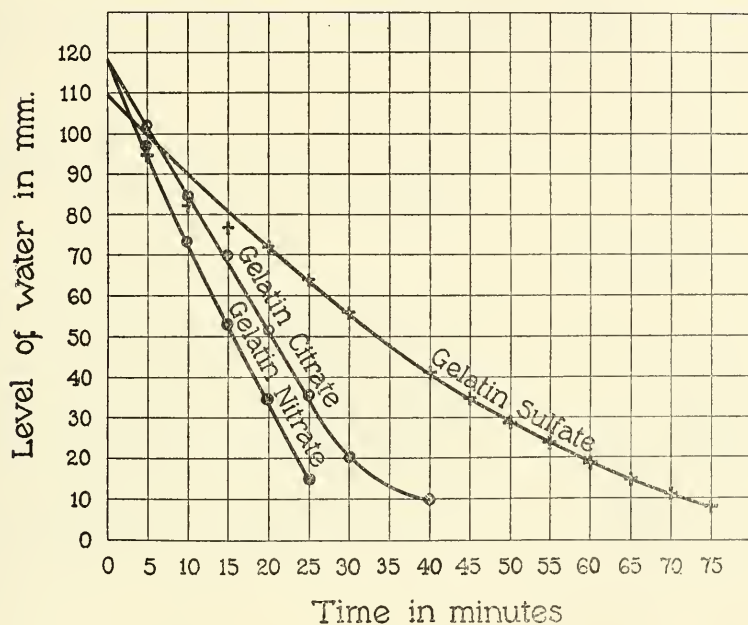


FIG. 5. Curves of fall of level of water when diffusing under an initial pressure head of about 120 mm. of H_2O against 1 per cent solutions of gelatin sulfate, gelatin citrate, and gelatin nitrate ($pH = 3.5$).

the manometer of the collodion flask. The initial pressure head was 125 mm. It required about 25 or 30 minutes for the pressure head of distilled water to fall from 120 mm. to about 20 mm. when it diffused into gelatin nitrate and monogelatin citrate, and 60 minutes when the distilled water diffused into gelatin sulfate.

The second method of testing the influence of the anion on the rate of diffusion of water consisted in determining the balancing

concentration of cane sugar for each of these salts in the way described. Table II gives the values observed after 30 minutes. The gelatin solutions had a pH of 3.5.

Using the same criterion as before we find that the balancing concentration of cane sugar lies for gelatin sulfate between $3M/32$ and $M/8$ and for all the others between $3M/16$ and $M/4$. In other words, the balancing concentration possesses for gelatin sulfate about one-half the value found for the other salts.

The writer has already published measurements of the osmotic pressure of 1 per cent solution of different gelatin salts. The osmotic

TABLE II.

Nature of gelatin salt used.	Change of level of liquid in the manometer tube of different concentrations of cane sugar solution when immersed in 1 per cent solutions of gelatin acid salts of pH 3.5, after 30 min.											
	Concentration of cane sugar solution.											
	M/1	3M/4	M/2	3M/8	M/4	3M/16	M/8	3M/32	M/16	M/32	M/64	
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.
Gelatin chloride.....	265	198	110	+15	+6	-18	-12	-22	-22	-19	-19	
“ nitrate.....	262	200	98	+25	+8	-11	-10	-19	-18	-19	-16	
Monogelatin oxalate.....	273	203	146	+75	+33	0	-4	-12	-17	-15	-20	
“ tartrate.....	288	215	159	+47	+30	-4	-6	-20	-14	-16	-19	
“ phosphate.....	290	196	125	+45	+18	-8	-14	-19	-14	-13	-16	
“ citrate.....	335	244	115	+53	+30	0	-5	-9	-16	-24	-11	
Digelatin sulfate.....	278	215	143	+74	+35	+10	+2	-8	-14	-14	-19	

pressure varies for the same concentration of a gelatin salt with the pH of the solution and is a maximum at a pH of about 3.4. It was found that the maximum osmotic pressure of a 1 per cent solution of gelatin chloride, gelatin bromide, gelatin nitrate, gelatin acetate, monogelatin oxalate, monogelatin tartrate, monogelatin phosphate, and monogelatin citrate is about 320 mm. of the gelatin solution; while for a 1 per cent solution of gelatin sulfate the highest pressure obtainable is about 130 mm. This is in satisfactory agreement with the ratio we should expect on the basis of the influence of the anions on the rate of diffusion of the negatively electrified particles of water.

We considered it necessary to obtain also the curves for the increase

in the height of the level of water in the manometer with time until the equilibrium is reached. 1 per cent solutions of gelatin chloride, nitrate, oxalate, tartrate, citrate, and sulfate were prepared by making up the solution with the addition of as much of these acids to 1 gm. isoelectric gelatin as was required to produce a pH of 3.5. These 1 per cent gelatin solutions were put into collodion flasks containing the glass tube as described and each flask was put into a beaker with 350 cc. of distilled water the pH of which was made 3.0 by adding in each case the same acid as that of the gelatin. In Fig. 6 is plotted the rise and the final osmotic pressure of these solutions. The osmotic pressure reached in the case of gelatin sulfate was about 159 mm. while for the other salts it was almost twice as high (between 260 and 310 mm.). It is also noticeable from the curves that the relative velocity of rise during the 1st hour of the experiment also showed about the same ratio of almost 1:2 as the final equilibrium. The results thus confirm our expectation that on account of the electrification of the water molecules both the rate of diffusion as well as the final osmotic equilibrium are affected in the same sense.

If we wish to explain the differences between the observed osmotic pressure of solutions of calcium and sodium gelatinate or of gelatin chloride and gelatin sulfate, exclusively on the basis of van't Hoff's law, we are compelled to seek refuge in the assumption of the formation of aggregates of gelatin ions by which the number of these particles is diminished without diminution of the number of their charges.¹¹ This suggestion was first offered by Bayliss¹² when he found that the osmotic pressure of the colloid Congo red was considerably lower than was to be expected according to the molecular concentration and the conductivity of the solution. This assumption, however, which the writer had also tentatively accepted to explain the difference between the osmotic pressure of calcium and sodium gelatinate of the same concentration rests only on the facts which the assumption is supposed to explain, while the connection between rate of diffusion of water and osmotic pressure of a solution holds generally in all

¹¹ The writer has shown that the conductivity of 1 per cent Na gelatinate and of 1 per cent Ca gelatinate at the same pH is identical.

¹² Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911-12, lxxxiv. 229.

cases of dynamical equilibrium between two processes which occur simultaneously in opposite directions; *e.g.*, chemical equilibrium between reversible reactions.

Procter¹³ and his collaborators have developed a theory of swelling of colloids based on the assumption that swelling is a purely osmotic phenomenon. On the basis of this theory we may consider the

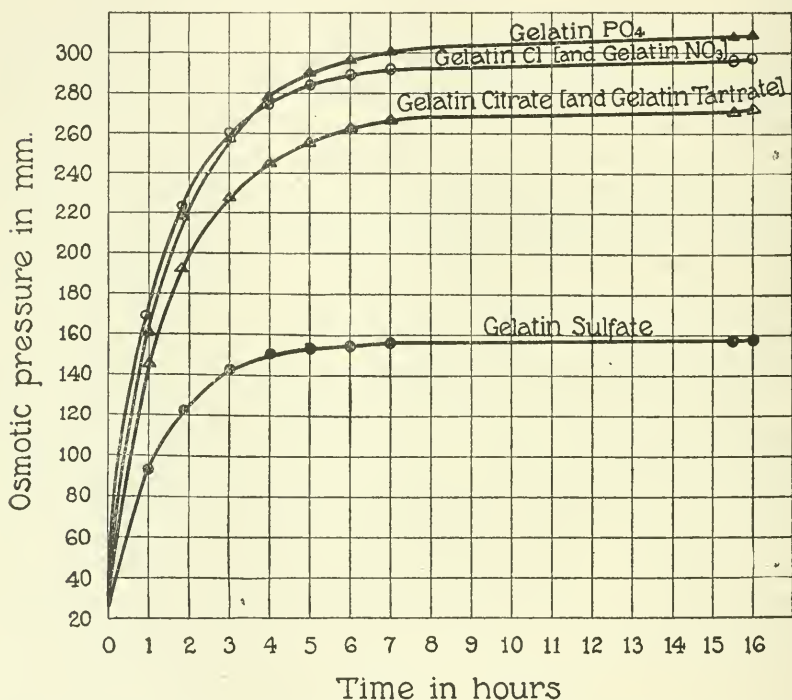


FIG. 6. Curves of osmotic pressure of 1 per cent solutions of gelatin phosphate, gelatin chloride (nitrate), gelatin citrate (tartrate), and gelatin sulfate (pH = 3.5). Pressure in mm. of column of gelatin solution.

surface and all the other solid parts of a block of gelatin to act as membranes permeable for water and crystalloids but not for gelatin. The osmotic conditions inside a solid block of gelatin which is

¹³ Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307. Procter, H. R., and Burton, D., *J. Soc. Chem. Ind.*, 1916, xxxv, 404.

submersed in water are therefore comparable to those in our experiments except that we substitute a collodion membrane for the gelatin membrane.

The writer has shown that metal gelatinates of the same concentration of gelatin and of hydrogen ions swell much more when the metal in combination with the gelatin is monovalent (*e.g.* Li, Na, K, or NH_4) than when it is bivalent (*e.g.* Ca or Ba).⁸ When gelatin is a cation the swelling is greater when the anion in combination with gelatin is monovalent (*e.g.* Cl, NO_3 , or H_2PO_4) than when it is bivalent (*e.g.* SO_4).¹⁰ The curves representing the influence of different acids and alkalies on the swelling of gelatin are similar to the curves representing the influence of the same acids and alkalies on the osmotic pressure of gelatin solutions. This similarity becomes clear if we adopt Procter's osmotic theory of swelling, adding to it our theory of the rôle of the electrification of particles of water in the phenomena of osmosis. In these experiments the solution surrounding the block of gelatin salt must have a low concentration of electrolyte since the presence of an excess of electrolyte suppresses the swelling, as pointed out in previous publications.

SUMMARY.

1. Amphoteric electrolytes form salts with both acids and alkalies. It is shown for two amphoteric electrolytes, $\text{Al}(\text{OH})_3$ and gelatin, that in the presence of an acid salt water diffuses through a collodion membrane into a solution of these substances as if its particles were negatively charged, while water diffuses into solutions of these electrolytes, when they exist as monovalent or bivalent metal salts, as if the particles of water were positively charged. The turning point for the sign of the electrification of water seems to be near or to coincide with the isoelectric point of these two ampholytes which is a hydrogen ion concentration of about $2 \times 10^{-5} \text{ N}$ for gelatin and about 10^{-7} N for $\text{Al}(\text{OH})_3$.

2. In conformity with the rules given in a preceding paper the apparently positively charged water diffuses with less rapidity through a collodion membrane into a solution of Ca and Ba gelatinates than into a solution of Li, Na, K, or NH_4 gelatinates of the same concentra-

tion of gelatin and of hydrogen ions. Apparently negatively charged water diffuses also with less rapidity through a collodion membrane into a solution of gelatin sulfate than into a solution of gelatin chloride or nitrate of the same concentration of gelatin and of hydrogen ions.

3. If we define osmotic pressure as that additional pressure upon the solution required to cause as many molecules of water to diffuse from solution to the pure water as diffuse simultaneously in the opposite direction through the membrane, it follows that the osmotic pressure cannot depend only on the concentration of the solute but must depend also on the electrostatic effects of the ions present and that the influence of ions on the osmotic pressure must be the same as that on the initial velocity of diffusion. This assumption was put to a test in experiments with gelatin salts for which a collodion membrane is strictly semipermeable and the tests confirmed the expectation.

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THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

VOLUME II, NO. 2

NOVEMBER 20, 1919



PUBLISHED BIMONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.
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Entered as second-class matter November 25, 1918, at the Post Office at Baltimore, Md., under the Act of March 3, 1879.

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THE HELIOTROPISM OF ONCHIDIUM: A PROBLEM IN THE ANALYSIS OF ANIMAL CONDUCT.

By W. J. CROZIER AND L. B. AREY.

(Contributions from the Bermuda Biological Station for Research, No. 108, and from the Physiological Laboratory, College of Medicine, University of Illinois.)

(Received for publication, July 21, 1919.)

I.

Onchidium floridanum is a small naked pulmonate which lives in holes and crevices of the shore between tides. A description of the bionomics of this mollusk we shall present more fully in another connection,¹ omitting here those aspects not bearing directly upon the most curious puzzle propounded by its behavior, which in brief is this: under natural conditions the movements of *Onchidium* take place in the light, but without reference to photic control; yet when the animal is tested apart from its natural surroundings it is found always to be negatively phototropic.

In view of this apparent incompatibility, in reality very deep seated, one is led to inquire: Is the behavior of *Onchidium* an instance where "unnatural conditions" imposed in phototropism experiments vitiate attempted analytic explanation of normal activities and more especially what, fundamentally, is the *sense* of the state of affairs we have outlined, in terms of adaptation?

II.

1. *Phototropism*.—Exploration of the dome-shaped dorsum of the mantle of *Onchidium* with a light-beam 0.3 mm. in diameter and of moderate intensity showed that the movements of the mollusk, in the laboratory could be directed at will by even as small an illumi-

¹ A preliminary statement of certain of the results, utilized for different purposes than in the present connection, is found in the papers: Arey and Crozier (1918) and Crozier and Arey (1919a).

61617-1919

nated area as 0.3 sq. mm. To accomplish this result it was necessary to stimulate the anterior end of the mantle, which is more irritable, photically, than other regions of the mantle. The animal contracts on the non-stimulated side, and creeps away from the source of activation. The whole mantle dorsum is photosensitive, local areas puckering sharply into depressed pockets, secondarily extending as furrows across the animal's back, when one spot is illuminated. The anterior end of the foot, and the oral lappets, are more sensitive in this respect.

The tentacles, which in some *Onchidia* carry terminal eyes, are non-reactive to light, nor are the orienting movements of the animal interfered with in any way by the removal of the tentacles.

It therefore appears that although, as we have ascertained, this species of *Onchidium* does not possess differentiated mantle eyes (Sempér, 1877; Stantschinsky, 1908), the physiological precursors, or perhaps the remnants, of such structures are actively functional in *Onchidium floridanum*.

Onchidium is quite unresponsive to increase of light intensity as such, but is very promptly and precisely oriented by incident light having a horizontal intensity component. There is found at all times and without exception a precise negative phototropism. That such orientation is in no way determined by "changes of intensity" is adequately demonstrated by the fact that these animals are at the same time conspicuously reactive to shading. When the light falling upon an *Onchidium* in air is suddenly decreased, the tentacles are forcibly withdrawn beneath the mantle, the head is retracted, locomotion stops, and the mantle is pressed into contact with the substratum. The distribution of this mode of irritability coincides with that evidenced in responses initiated by illumination. As with *Holothuria*, *Chiton*, and some other forms, the nature of the photic orientation of *Onchidium* is profoundly inconsistent with the differential sensitivity exhibited by the same animal (Crozier and Arey, 1918).

2. *In the Field*.—*Onchidium* lives during high-tide in "nests," cavities in the rock containing a number of individuals, from which the mollusks at low water emerge to feed upon exposed shore surfaces. The individuals emanating from any one nest return simultaneously to that nest before the tide rises again (Arey and Crozier, 1918).

The natural wanderings of the *Onchidia* while feeding upon the rocks take place without reference to the nature of the sunshine, whether brilliant or dull, and bear no relation whatsoever, at any time, to the direction of the incident light. The *Onchidia* creep out from their sheltering cavities only during daylight hours, however, and never at night, no matter how bright the moon.

In the laboratory, as far as our numerous experiments have shown, *Onchidium* moves away from a source of light under any conditions of temperature (15–32°), dryness (under water, or in air), and light (regardless of intensity) compatible with its active existence; whereas, on its natural substratum the same individual may creep directly into the horizontal rays of the setting sun, or away from them, with indifference. This state of affairs is well exemplified by the fact that, if an *Onchidium* is picked up from its natural substratum and a glass plate slipped between it and the rock, the animal orients immediately away from the sun, and with machine-like precision. Mere disturbance is not responsible for this phenomenon, because an equivalent handling of the mollusk, followed by its replacement on the rock, does not lead to the exhibition of negative heliotropism (provided the animal is restored to the rock within a certain radius of its “nest”).

Similar results are obtained if an *Onchidium* creeping on the shore is shaded from the sun and then reilluminated from a new direction by light reflected from a mirror. Momentary “hesitation” may succeed such illumination, but the creature’s path is not materially influenced.

3. *Inhibition of Heliotropism.*—We would emphasize the point that the natural movements of *Onchidium* cannot be viewed as “contrary to the dictates of the animal’s heliotropism;” but that *during the creeping of the mollusk on the rock surface immediately surrounding its “home” this heliotropism is completely inhibited.* Proof of the correctness of this contention is seen in these facts, each of which has been verified upon a number of occasions.

(a) Removal of the oral lappets of an *Onchidium*, organs which normally are in constant contact with the substratum, or their anesthetization by MgSO_4 , obliterates the normal directed homing of the animal; at the same time its negative heliotropism becomes a dominant factor in the control of creeping.

(b) An *Onchidium* removed to a strange section of shore moved about in a manner largely or entirely directed by the illumination. The same is true of any *Onchidium* which has been kept in an aquarium for 24 hours and is then returned to its native scene.

(c) In favorable instances, injection of *Onchidia* with strychnine (0.1 to 0.2 cc. of 1 per cent strychnine hydrosulfate), while not producing more than a moderate temporary contraction, succeeded by normal creeping, leaves the snail at the mercy of its heliotropism, although the animal may not have been handled at all, the injecting needle being simply inserted in its back and discharged.

These facts make it evident that in nature, as far as directed progression is concerned, the heliotropism of *Onchidium* is simply inhibited, or suffers central block, probably owing to those guiding impulses originating in the substratum and mediated through the oral lappets. Strychnine, while not necessarily "converting inhibition into excitation" (Cushny, 1919; Arey and Crozier, 1919), lowers the central threshold of solar activation impulses.²

It is improbable, furthermore, that the diurnal rhythmicity of *Onchidium's* appearance from its concealed rock cavities has any relation to heliotropism. The tidal rhythm of appearance is such, as we have learned by long observation, that even (as in summer) though two periods of low water may occur during daylight hours, the *Onchidium* colonies creep out to feed but once in the 24 hours. On the other hand, especially in winter,³ several days may pass before a tidal period occurs in good daylight; the *Onchidia* then may make no appearance for several days. The brightness or dullness of the day has probably, as far as we can detect, no influence on emergence.

III.

With reference to the adaptation of habits to scene of life, the other activities of *Onchidium* are no less curious than its directed creepings ("homing") on the shore. It might be said that it is to the best interests

² "Reversals" of an analogous sort, but normally determined through feeding activities, are possibly involved in "homing" movements; this matter we shall treat separately.

³ *O. floridanum* does not hibernate, as some other species appear to do (Hirasaka, 1912) in colder climates.

of this animal to come out into the sunlight; it must do so in order to obtain food; therefore, it disregards the dictates of its negative phototropism. This notion is sufficiently disposed of in the foregoing section; the negative heliotropism of *Onchidium* is not "disregarded" normally, it is centrally inhibited by other impulses competing for the nervous control of the body musculature. The further question remains: What is the rôle of the heliotropism of *Onchidium*? The mere existence of this type of response is sufficiently accounted for by the composition of the animal and the structural disposition of its parts. We have to inquire if this heliotropism is ever of "use."

Note in the first place our finding that the movements of an *Onchidium* on a rock substratum remote from its own "nest" are largely, and, in many instances, exclusively determined by the position of the sun. Might it not then be supposed that if an *Onchidium* should accidentally suffer displacement from its usual surroundings, its negative orientation by light would force it to enter some crevice or rock cavity, thus securing shelter and perhaps membership in a new colony? But the facts are different. The natural displacement of an *Onchidium* in this way is probably of very rare occurrence; these snails possess a well disposed system of repugnatorial glands (Crozier and Arey, 1919a), which makes such an event unlikely. Much more serious, however, is the fact that the animal withdraws sharply when shaded, as already stated. Furthermore, numerous instances we have carefully watched have yielded not one good case in which an *Onchidium* would enter a strange "nest" and remain there for more than a few minutes near the entrance (Arey and Crozier, 1918); they always crept out and away. These two kinds of response make it improbable that a "lost" *Onchidium* would seek refuge in a strange depression, and those which were purposely displaced in this way remained in fact creeping about on the rock until washed off by the rising tide.

IV.

We must refuse, then, to admit any obvious adaptive significance inhering in the photic behavior of *Onchidium*. Loeb has pointed out (1916) that positive heliotropism occurs in several arthropods "which have no opportunity to make use of it." Instances of this

kind have drawn from some writers the curious comment that "these reactions are non-adaptive only under artificial conditions" (Mast, 1911), with the further assumption that "the reactions were inherited from ancestors in which they were adaptive." The normal inhibition of heliotropism in *Onchidium* is adaptive, if you like, but the further difficulty of finding some adaptive excuse for the existence of heliotropism at all remains all the more insoluble. The behavior of *Onchidium* is more curious than that of the crustaceans cited by Loeb, for there is physical opportunity to "make use" of their photic sensitivity, since they are exposed to light. Whether the mechanism for such a response is merely an "inheritance" from ancestors in which it was adaptive, one need not say, but the mechanism is fully present, though seemingly not used, and of obscure, if any, functional value—as far as concerns the movements of *Onchidium* on the rock surfaces.

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THE COMBINATION OF ENZYME AND SUBSTRATE.

I. A METHOD FOR THE QUANTITATIVE DETERMINATION OF PEPSIN.

II. THE EFFECT OF THE HYDROGEN ION CONCENTRATION.

BY JOHN H. NORTHROP.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 7, 1919.)

I. A Method for the Quantitative Determination of Pepsin.

Considerable experimental evidence has been obtained by various authors to show that enzymes are removed from their solutions by insoluble substances.^{1,2} Pepsin has been especially studied from this view-point. Dauwe³ showed that this property of removing pepsin from its solution was not common to all substances and was connected in some way with the digestive action of the enzyme. He was also able to show that the size of the particles, at least in the case of egg albumin, was without any marked effect on the amount of pepsin removed.

Abderhalden⁴ and his coworkers state that pepsin is removed almost completely from its solution by insoluble proteins on which it acts and they consider that this plays an important rôle in the kinetics of the reaction. These results were partially confirmed by Leary and Shieb.⁵

¹ For the literature see Bayliss, W. M., *The nature of enzyme action*, London, 3rd edition, 1914.

² Nelson, J. M., and Griffin, E. G., *J. Am. chem. Soc.*, 1916, xxxviii, 1111.

³ Dauwe, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 426. This paper reviews the earlier work.

⁴ Abderhalden, E., and Steinbeck, E., *Z. physiol. Chem.*, 1910, lxviii, 293. Abderhalden, E., and Strauch, F. W., *ibid.*, 1911, lxxi, 315. Abderhalden, E., and Wachsmuth, F., *ibid.*, 339. Abderhalden, E., and Friedel, F., *ibid.*, 449. Abderhalden, E., and Kramm, F., *ibid.*, 1912, lxxvii, 462.

⁵ Leary, J. T., and Shieb, S. H., *J. Biol. Chem.*, 1916-17, xxviii, 393.

Bayliss¹ attaches considerable importance to the combination of enzyme and substrate and considers it an essential point in the theory of enzyme action. Van Slyke and Cullen⁶ were able to formulate the kinetics of enzyme action on the basis of the law of mass action by assuming the existence of a compound between enzyme and substrate.

The study of this combination, however, has not furnished any quantitative experimental data as to the nature of the reaction or the influence of various factors on it—due largely to the difficulty of determining quantitatively the amount of enzyme.

It seemed important, therefore, to obtain quantitative experimental data on this subject. In order to do this it was necessary to have a convenient and accurate method for the determination of pepsin. It was found that the change in the conductivity during the digestion of egg albumin by pepsin afforded such a method. Sjöqvist⁷ found that there were marked changes in the conductivity during pepsin digestion. His results were confirmed with the exception that the change was found not to follow the actual rate of digestion. It can be used therefore only as an empirical method for the determination of pepsin and not for the study of the kinetics of the reaction, as was done by Sjöqvist. The reason for this divergence is probably due to the fact that the change in conductivity is due to two causes; (1) the liberation of free acid (carboxyl) groups which increase the conductivity, and (2) the liberation of free amino groups which bind some of the free acid and so decrease the conductivity. This explanation is borne out by the following facts.

With dilute solutions of egg albumin containing strong acetic acid (pH 2.3) there is a regular small increase in the conductivity which nearly parallels the increase in free amino groups as followed by the increase in amino nitrogen. This is due to the fact that, owing to the very large excess of free undissociated acid present, the ions which are removed from solution by combination with the free amino groups are replaced by the dissociation of more acid, and so kept nearly constant. The slight increase in conductivity is therefore due to the liberation of free carboxyl groups. In strong acid solu-

⁶ Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.

⁷ Sjöqvist, J., *Skandin. Arch. Physiol.*, 1893-95, v, 277.

tions, however, as hydrochloric, there is a rapid decrease in the conductivity—due to the removal of acid ions by combination with the liberated amino groups. This change is so much larger than the increase due to the acid groups set free by the protein that the increase in conductivity due to the latter is more than compensated. It has already been shown⁸ that the actual rate of digestion is approximately the same in all acids at the same reaction so that the differences in the changes in the conductivity cannot be ascribed to differences in the rate of digestion.

It was found that the maximum change occurs in strong solutions of egg albumin titrated to pH 2.6 with hydrochloric acid. This solution was therefore used. The conductivity was followed by means of the apparatus described by Taylor and Acree.⁹ The electrodes were of the dipping type and were immersed in the solution in order to make a reading. It was found that the percentage change in conductivity was constant for a given quantity of pepsin, irrespective of the absolute value of the original conductivity. The readings and figures are therefore given in terms of the increase in the scale readings on the bridge, which for small readings are equivalent to the percentage change. The measurements were carried out as follows.

25 cc. of a 3 per cent egg albumin solution were pipetted into a series of large "Non-sol" test-tubes and suspended in a water bath at $37^{\circ} \pm 0.02^{\circ}$. The electrodes were immersed in the solution and 1 cc. of the pepsin solution was added. The external resistance was then set so as to give a bridge reading of 500; *i.e.*, the middle of the bridge. The change in conductivity was now followed by the bridge readings. These figures are related to the actual change in resistance of the solution by means of the formula $\frac{X}{R} = \frac{A}{1,000 - A}$ where X = resistance of the solution, R is the external resistance, and A is the bridge reading.

The figures given in Table I are the increase in the value of A and are very nearly proportional to the percentage increase in the resistance.

⁸ Northrop, J. H., *J. Gen. Physiol.*, 1918-19, i, 607.

⁹ Taylor, W. A., and Acree, S. F., *J. Am. Chem. Soc.*, 1916, xxxviii, 2396.

Readings were taken at intervals so as to give points corresponding to changes of 2 to 4 units of the bridge reading (which could be easily read to 0.25 units). These points were then plotted on a large scale by means of a flexible "spline" and weights, so that the curves were

TABLE I.

Change in Conductivity of Solution of Egg Albumin with Varying Amounts of Pepsin.

25 cc. of egg albumin solution, pH 2.6. *R* 320.

Temperature $37^{\circ} \pm 0.02^{\circ}\text{C}$. *A* at beginning 500.

Relative amount of pepsin added in volumes of 1 cc.							
100		50		25		12.5	
Time.	Increase in scale reading.	Time.	Increase in scale reading.	Time.	Increase in scale reading.	Time.	Increase in scale reading.
<i>min.</i>		<i>min.</i>		<i>min.</i>		<i>min.</i>	
3	4.0	4	3.0	3	1.5	7	2.2
8	8.5	13	7.8	17	6.0	9	2.7
14	12.0	23	11.0	28	8.0	26	5.2
18	14.5	39	16.0	46	11.5	34	6.7
31	21.0	51	19.5	60	13.5	67	9.2
43	26.0	88	26.5	93	18.5	102	13.2
83	38.0						

Change.		Time necessary to cause change.		
Increase in scale reading.	Time.			
	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
5 to 10	6.0	11.8	22.4	44.0
10 to 15	8.1	14.4	32.0	

Interval.	Relative amount of pepsin calculated.			
5 to 10	(100)	51	27	13.6
10 to 15	(100)	56	25	
Average.	(100)	53	26	13.6

30 to 50 cm. long. The time necessary to cause a given change was then interpolated from these curves. In this way errors in individual readings were smoothed out and, as the curves are regular, accurate values for the time necessary to cause a given change could be ob-

tained. Since there is always some uncertainty about the zero reading the interval from 0 to 5 was omitted and the time to cause a change of from 5 to 10 and from 10 to 15 units was taken. The values obtained in this way were then averaged and the result taken as proportional to the reciprocal of the amount of pepsin present. By comparing this figure with the corresponding one from a control containing a standard amount of pepsin, the relative quantity of pepsin present could be determined, since it was found that the time

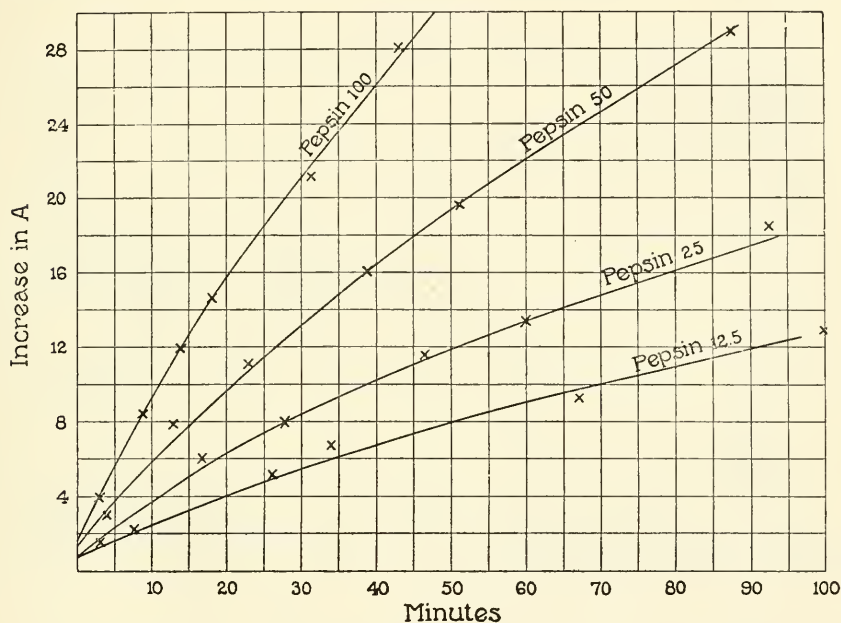


FIG. 1.

necessary to cause any given change was inversely proportional to the amount of pepsin present. That is, double the quantity of pepsin requires half the time, etc. (Arrhenius' "QT" rule).¹⁰

Table I and Fig. 1 give the results with a series of solutions containing different amounts of pepsin. It will be seen that the amount of pepsin can be determined with an accuracy of ± 2 to 3 per cent,

¹⁰ Arrhenius, S., Quantitative laws in biological chemistry, London, 1915.

and further that ten to fifteen determinations can be made in the course of 1 to 2 hours depending on the amount of pepsin present.

It was now possible to determine quantitatively the amount of pepsin removed from solution by various substances.

Table II shows the results of such a series with coagulated and dried egg albumin. The control experiments show that the decrease in the amount of pepsin is not due to the destruction of the pepsin on standing or to the retarding effect of the products of reaction.

TABLE II.

Change in Concentration of Pepsin in Various Solutions.

Temperature 25°C.

pH of all solutions, 2.5.

1 cc. of solution pipetted off and pepsin estimated at time noted.

Time.	Relative amount of pepsin per cc.		
	20 cc. of pepsin solution.	20 cc. of pepsin solution + 1 gm. of egg albumin in solution.	20 cc. of pepsin solution + 1 gm. of coagulated egg albumin.
<i>min.</i>			
1	(100)	101	95
10	101	101	84
20	94	96	82
40	95	100	86
80	100	128*	80
160	97	98	86

* This is an experimental error. The curve was irregular and gave widely divergent results for different intervals.

A series of experiments was now made with various substances. The results are summarized in Table III. It is obvious that the removal of the pepsin is not purely a matter of surface but that it is dependent in some way on the substance itself.¹¹

This fact is brought out more strongly in the experiments summarized in Table IV and Fig. 2, where coagulated egg albumin of different sized particles was used. It is evident that the equilibrium

¹¹ The author does not doubt the existence of adsorption or concentration in the surface layer in the sense of Willard Gibbs. This phenomenon, however, is evidently of subordinate importance here.

reached is independent of the size of the particles and therefore of their surface. These experiments were repeated under slightly different conditions several times—always with the same result. This would indicate that the process is either one of solution, in which

TABLE III.

Removal of Pepsin from Solution by Different Substances.

10 cc. of pepsin solution, pH 2.5, + 0.5 gm. of substances noted. Allowed to stand 10 min. at 25°C. and pepsin estimated in 1 cc. of solution.

Substance.	Relative quantity of pepsin per cc.
Control; pepsin solution alone.....	100
Starch.....	98
CaSO ₄	86
Agar.....	103
Kaolin.....	100
Blood charcoal.....	15
Casein.....	70
“ (coagulated, dried, and ground to 40 mesh)	74
“ (extracted with boiling alcohol for 24 hrs.).....	60
“ C (charred at 150°C.).....	90
Egg albumin (coagulated, dried with acetone, and finely powdered)	10

TABLE IV.

Effect of Size of Particles of Egg Albumin, Coagulated, Dried, and Ground.

2.0 gm. in 20 cc. of pepsin solution titrated to pH 2.5 + HCl.

1.0 cc. pipetted off and analyzed for pepsin at time noted.

Time.	Size of particles.	
	Through 10 mesh but not through 20 mesh.	Through 80 mesh.
	Relative amount of pepsin per cc.	
<i>min.</i>		
1		46
2	98	21
4	93	30
8	72	12
30	32	10
60	12	14
120	10	11

case the law of partition coefficients should be found to hold, or of chemical combination, in which case the law of mass action should apply. Preliminary experiments indicate that the process follows the law of partition coefficients.

It may be objected that the effective surface is not the actual surface of the particles but some fine interior structure which is the same in all. The particles, however, swell in acid and appear translucent and homogeneous. It would seem that any interior surfaces must be of nearly intermolecular dimensions. In this case, of course, all chemical phenomena may be considered "surface" phenomena.

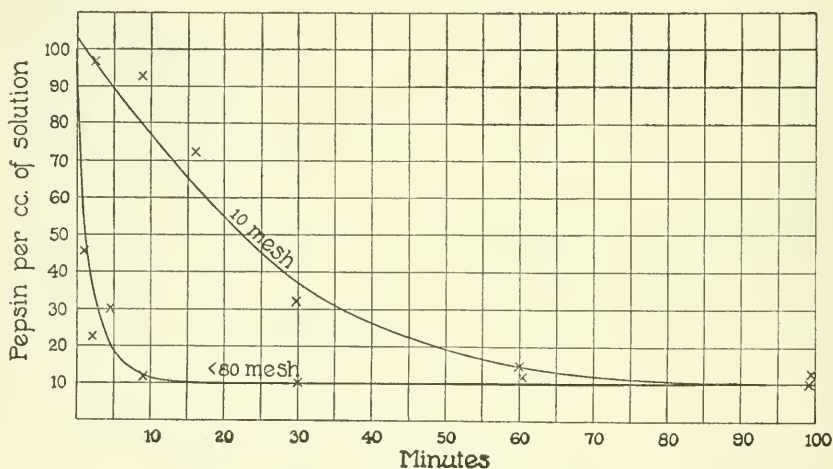


FIG. 2.

II. The Effect of the Hydrogen Ion Concentration.

Inasmuch as the activity of pepsin is dependent to a large extent on the hydrogen ion concentration it appeared probable that the combination of pepsin with its substrate would be a function of the same variable. Table V and Fig. 3 show the results of a series of experiments made at different hydrogen ion concentrations. There is a decided optimum for the combination of pepsin with its substrate corresponding (within the limits of error) to the optimum for digestion. These experiments were repeated with casein with approximately the same results. The optimum zone for the digestion of proteins

by pepsin therefore is due to the fact that at this degree of acidity more pepsin combines with the protein than in either a more or less acid solution. Van Slyke and Zacharias,¹² from a study of the constants of their equation for the action of urease, decided that the hydrogen ion concentration affected the *rate* of combination of the

TABLE V.

Effect of Reaction of Solution on Combination of Pepsin and Coagulated Egg Albumin.

Experiment A.

Temperature 25°C.

0.5 gm. of egg albumin suspended in 10 cc. of HCl of increasing strength. 1.0 cc. of strong pepsin solution added. Tube shaken, allowed to stand 1 min., and clear liquid pipetted off. pH measured (by gas chain) in part of this sample. 5.0 cc. of remainder brought to same reaction in all tubes by addition of a few drops of strong HCl. All brought to same volume with water and pepsin estimated in 1 cc.

Experiment B.

Same as A, but allowed to stand 2 min.

pH of solution.	Relative amount of pepsin per cc.		No albumin. (Control.)	Relative amount of pepsin combined.	
	Experiment A.	Experiment B.		Experiment A.	Experiment B.
0.88	77		100	23	
1.00		79			21
1.24	69			31	
1.3		68			32
2.08	60		100	40	
2.2		50			50
2.75	63			37	
2.8		47			53
3.5	64			36	
3.9		68			32
4.4	77	80		23	20
4.9	100	86	100	0	14
5.7	100	94	100	0	6

enzyme and substrate. In the case of pepsin, however, it is not the *rate* of combination but the *amount* which is influenced. This is shown by the fact that little or no pepsin is removed from its solution by its substrate at a reaction of 5.0, no matter how long they are

¹² Van Slyke, D. D., and Zacharias, G., *J. Biol. Chem.*, 1914, xix, 181.

left in contact. It seems probable that this is due to a change in the condition of the protein rather than to a change in the enzyme, since, according to Ringer,¹³ the optimum reaction is different for different proteins.

The simplest explanation of the above facts would seem to be that the quantity of ionized protein present determines the amount of pepsin which combines with the protein, and hence also determines the rate of digestion. Some direct evidence confirming this hypothesis has been obtained and will be discussed fully later.

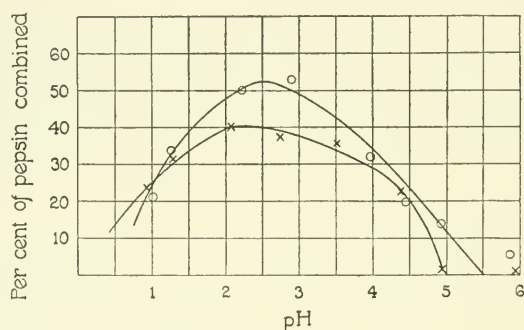


FIG. 3.

SUMMARY.

1. A quantitative method for the determination of pepsin is described depending on the change in conductivity of a digesting egg albumin solution.
2. The combination of pepsin with an insoluble substrate has been followed by this method.
3. The amount of pepsin removed from solution by a given weight of substrate is independent of the size of the particles of the substrate.
4. There is an optimum zone of hydrogen ion concentration for the combination of enzyme and substrate corresponding to the optimum for digestion.
5. It is suggested that the pepsin combines largely or entirely with the ionized protein.

¹³ Ringer, W. E., *Kolloid-Z.*, 1916, xix, 253.

LABYRINTH AND EQUILIBRIUM.

I. A COMPARISON OF THE EFFECT OF REMOVAL OF THE OTOLITH ORGANS AND OF THE SEMICIRCULAR CANALS.

By S. S. MAXWELL.

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(Received for publication, September 16, 1919.)

INTRODUCTION.

According to the conceptions of Mach and Breuer we must distinguish two kinds of equilibrial functions in the ear; the one, dynamic through which movements of rotation are perceived, and the other, static by which is produced a definite orientation or sensation of position in relation to the lines of gravitational force. It was suggested that the dynamic function is performed by the sensory structures of the semicircular canals, the static by the otolith organs of the vestibule. Although this view has been widely accepted the literature of the subject is full of contradictions and the experimental evidence is far from satisfactory.

Results of experiments on the labyrinth on which I have been engaged for a long time show that no sharp differentiation exists between the functions of the otolith organ of the utriculus and the ampullæ of the semicircular canals. I have found on the one hand that a labyrinth from which the ampullæ have been removed without injury to the vestibular portions possesses both dynamic and static functions, and that on the other hand a labyrinth from which the otolith organs have been removed without injury to the ampullæ retains both static and dynamic functions.

The experiments reported in this article have been made on dog-fish of the genera *Mustelus* and *Squalus*. The advantages of the dog-fish as an experimental animal have often been pointed out. Among these are the large size of the various parts of the labyrinth, the

clearness with which operations may be performed through the transparent cartilage of the skull, and the fact that bleeding can be almost wholly avoided and that the fish recovers readily from the shock of the operation.

In my experiments it has been necessary to use as indices of results the well known compensatory movements which occur on rotation, the righting reaction, and the general state of equilibrium. The compensatory movements of the eyes and fins of the dogfish, as first described by Loeb,¹ are remarkably constant. I have made especial use of the eye movements which may be described briefly as follows: if a dogfish is held in its normal resting position, belly down, and longitudinal body axis horizontal, the eyes assume a characteristic resting position, symmetrical to the body. This position of the body I shall refer to as normal and the position of the eyes as the primary position. If the animal is rotated to the right around its longitudinal axis, the eyes make complementary movements of such character that they tend to preserve their original position in space; that is, the right eye is elevated and the left eye is depressed. If the rotation is around the transverse axis, head inclined downward, both eyes appear to rotate on their axes by a wheel-like movement so that the anterior pole of each eye is elevated and the posterior pole is depressed. If the animal is rotated backward around its transverse axis both eyes make a compensatory wheel-like forward rotation. If the animal is rotated about an obliquely placed horizontal axis the compensatory movement is a resultant of the effects which would be produced by the rotations around the two axes of reference. These compensatory movements are made during the rotation, but if the abnormal body position is retained, the complementary position of the eyes is also retained. It is seen, of course, that as long as the animal is kept in the abnormal position there is a constant force; namely, the force of gravity, acting upon its body elements in an unaccustomed direction.

If the animal is held in the normal position and the body is then rotated around its dorsoventral axis, both eyes turn by a conjugate movement in the opposite direction; that is, if the head is swung to the right both eyes turn to the left, and if the head is swung to the

¹ Loeb, J., Ueber Geotropismus bei Thieren, *Arch. ges. Physiol.*, 1891, xlix, 175.

left both eyes look to the right. If in these rotations the new position is retained, the new position of the eyes is not retained; they return to their primary position. It is seen at once that in the new position there is no altered relation to the lines of gravitational force.

Removal of the Ampullæ.

The ampullæ of the anterior vertical and the horizontal canals open into the utricle so near the macula acustica of the latter that most experimenters seem to have had difficulty in destroying the one set of structures without injury to the other. Lee² and Lyon³ each speak of destruction of the ampullæ, but both seem to have relied on section of the nerve branches. By section of the nerve branches, however, they arrived at exactly opposite and fundamentally contradictory results.

After considerable practice I have developed a special technique by which the ampullæ of any or all of the canals may be removed with a minimum of injury and shock to the animal and with results which admit of no uncertainty. A flap of skin is loosened and turned back exposing the appropriate portion of the skull. A thin surface layer of the skull is sliced off with the attachment of some of the neck musculature, thus making visible the parts of the labyrinth through the transparent cranial cartilage. The membranous canal is exposed at a distance not too great from its ampullar enlargement. With a fine pointed pair of curved forceps the membranous canal is grasped as closely as possible to the ampulla and the canal with its ampulla is extracted by a sudden movement, a light quick jerk. Success in this operation depends mainly upon the choice of forceps with the proper curve which bite at the very point, and upon acquiring the knack of removal of the canal by a suitable movement. A too sudden pull will usually break off the canal external to the ampulla, and too slow a movement frequently drags and injures portions of the vestibular structures which it is desired to leave unharmed. When

² Lee, F. S., A study of the sense of equilibrium in fishes, *J. Physiol.*, 1893, xv, 311.

³ Lyon, E. P., A contribution to the comparative physiology of compensatory motions, *Am. J. Physiol.*, 1899-1900, iii, 86.

one has once acquired the knack of this operation the results become absolutely clear. The ampullæ can be extracted one after another with certainty and exactness. In sectioning the nerves one may cut too much or too little; the fiber bundles are scattered, and certainty is impossible. The attempted destruction of the ampullæ *in situ* cannot by any means have the exactness of their complete removal. In many of my earlier experiments I had the ampullæ pasted on a blank leaf of my note book when I wrote down on the same page the results of their extirpation. Under these conditions there can be no doubt as to the correctness of the results. In the summer of 1919, I repeated and extended these experiments at the Marine Biological Laboratory, and on account of the contradictions of previous workers I took occasion to have the experiments witnessed by a number of physiologists and zoologists.

I had found⁴ that removal of the ampullæ of the four vertical canals had little or no effect on the compensatory eye movements resulting from the rotation around the longitudinal and transverse axes. In order, however, that there could remain no possible functioning of the semicircular canals I have in a long series of animals removed all six ampullæ with uniform results.

A dogfish from which all six ampullæ have been removed shows definitely the following reactions. (1) Compensatory movements of the eyes and fins occur on rotation around a longitudinal axis; *e.g.*, on rotation to the right, the right eye goes up and the left eye goes down. This position of the eyes is retained as long as the abnormal body position is continued. (2) Compensatory movements of eyes and fins occur on rotation around the transverse axis; *e.g.*, when the animal is tilted head downward the eyes make the characteristic wheel-like backward rotation. (3) Compensation is absent on rotation around the dorsoventral axis. (4) The animal swims in a manner differing but little from the normal. (5) The righting reaction takes place promptly and vigorously; if the animal is placed belly up in water it turns over at once.

As a sample experiment I quote *verbatim* the following from my notes.

⁴ Maxwell, S. S., Experiments on the functions of the internal ear, *Univ. California Pub. in Physiol.*, 1910-15, iv, 1.

"July 14, 1919. Dogfish 5. 10.00 a.m. All six ampullæ removed. Compensatory movements prompt on rotation around longitudinal and transverse axes; none on rotation in horizontal plane. Animal rights itself perfectly in water. Eyelids sewed together to exclude retinal stimuli, and animal put into deep tank; righting perfect.

2.00 p.m. Animal rather weak but rights itself promptly when turned over in water; swims rather wobbly; turned completely over once when excited by other dogfish; I have seen a normal dogfish do this under similar circumstances.

July 15, 9.00 a.m. Animal very weak; rests on bottom of tank in normal position. Rights itself but may swim one or two turns belly up before getting over. Opened stitches in eyelids. No compensatory movements of eyes.

July 16, 9.30 a.m. Animal moribund. Killed for autopsy. Considerable blood clot in each vestibule."

The above experiment shows a possible source of the confusion in the reports of previous investigators. Had I assumed that on account of shock effects observations made on the day of operation would be unreliable, and had I waited until the following day to make my observations, it would have appeared that loss of the ampullæ abolishes compensatory movements, which is manifestly not true. When immediately following the destruction of an organ a function is clearly retained it is indisputable proof that at least that organ is not the only one which can perform the function. Observations made on July 15, on Dogfish 5, might have favored the statement that destruction of the ampullæ of the semicircular canals abolishes compensatory movements of the eyes, but the observations of July 14 clearly show such a conclusion to be wrong.

In the attempts to determine the rôle of the various sense organs in the geotropic reactions of the dogfish, it has long been recognized that retinal stimuli play a part. Lyon³ and Parker⁵ excluded visual stimuli by section of the optic nerve. I have accomplished the same result by the less radical operation of sewing the eyelids together, when equilibrium and the righting reactions were under consideration, and by placing a black, opaque disk on the cornea over the region of the pupil when eye movements were to be studied. Other methods of blinding were also used. I can affirm with complete assurance

⁵ Parker, G. H., Influence of the eyes, ears, and other allied sense organs on the movements of the dogfish, *Mustelus canis* (Mitchill), *Bull. Bureau of Fisheries*, 1909, xxix, 43.

that the compensatory motions described in the case of animals from which all the ampullæ have been removed occur also when activity of the retina has been excluded.

It must also be noted that the dogfish, like most animals which rest on the bottom and are not merely suspended in the water, manifests very strong contact reactions. A vigorous specimen which has been blinded and which has had as far as possible all the end organs of the eighth nerve destroyed will almost always be found belly down when at rest. Such a fish may swim indifferently back or belly up, but when it comes to rest the position is a fair index of the general state of the animal. When an investigator affirms that his specimen came to rest indifferently in any position, he has given good incidental evidence as to the animal's physical condition. In stating that a dogfish deprived of its six ampullæ makes normal righting reactions I have not been unmindful of these facts, but have taken care to exclude the possibility of contact stimuli.

Although it can be proved that after the loss of all the ampullæ, with exclusion at the same time of retinal and contact stimuli, the dogfish makes normal compensatory movements of the eyes and fins to rotations in all vertical planes, it is necessary to note that there are some differences between this and a normal animal.

The following seem to be fairly constant results. (1) The compensatory movements of the eyes, though prompt, are noticeably slower than in the uninjured animal. Compensatory movements due to visual stimuli alone are so much slower, requiring several seconds or even minutes, that no difficulty is experienced in distinguishing these from reflexes of labyrinthine origin. (2) If seized while in the water the animal strongly resists the attempt to turn it back downward. One feels, however, that the resistance is neither as prompt nor as strong as in a normal animal. (3) In swimming there is more or less evident a slight tendency to sway from side to side around the longitudinal axis, like a boat insufficiently ballasted.

These three conditions are less noticeable in vigorous specimens; they become very marked in weakened individuals. They can perhaps all be accounted for by a lowering of muscle tonus. It is important to note that, as I shall show later, precisely the same complex of conditions can be brought about through a totally different operation.

Removal of the Otoliths.

In the dogfish the otoliths are of soft, friable, calcareous material. In the sacculus there is a large otolith spread over the main macula acustica and a smaller mass on the lagena. These are so situated that their removal can be accomplished with little injury and the operation is relatively easy. For the otolith of the utriculus the case is very different. This otolith lies in the recessus utriculi so close to the openings of the ampullæ of the anterior vertical and the horizontal canals that it requires some skill and much practice to remove it without injury to the ampullæ. If, however, after opening the vestibule by removing a portion of the cartilaginous roof, the utricular wall is slit open with a very sharp microdissection knife, the otolith material may be washed out by the careful use of a fine pointed pipette. In a similar way the saccule may be slit open and its otoliths removed. No operation was considered successful unless it was found at postmortem examination that no otolith material remained. For reasons to be stated in another paper it was considered important not only to avoid injury of the ampullæ but also to reduce the injury of the utriculus to a minimum.

After removal of all the otoliths from both ears in successful cases the following results are seen. (1) Compensatory movements of the eyes are made in the regular way to rotations about all three body axes, longitudinal, transverse, and dorsoventral. If the animal is rotated around a longitudinal or transverse axis and held in the abnormal position the compensatory position of the eyes is retained, but when the rotation is around the dorsoventral axis the eyes make the compensatory movement and then return to the primary position. These movements appear to differ from those in the normal animal only in being slightly slower. (2) The animal swims in normal orientation and maintains its equilibrium in the water, but its swimming, like that of the fish without ampullæ, is likely to be accompanied by a rocking movement; this rocking or swaying is less apparent in vigorous specimens. (3) If turned belly up in the water, the fish rights itself promptly; in doing so, however, it sometimes overcompensates and turns almost or completely over.

It will be seen that these results are strikingly similar to those

produced by loss of the ampullæ. It is especially noticeable that there is the same apparent slight slowing of the reactions and the same indication of lowering of muscle tonus in general. In one important respect, however, the result of this operation differs from that of removal of the ampullæ; namely, the compensatory movement to rotation about the dorsoventral axis is retained. It should be stated that in these observations due care was taken to eliminate retinal and contact reactions.

Parker⁵ removed the saccular otolith by way of an opening in the roof of the mouth. He found that the loss of this otolith alone produced no noticeable effect on the equilibrium or the righting reactions of the dogfish, nor did there appear to be any loss of tonus. I have removed this otolith many times by the method I have described above. Its loss does not alter or weaken any of the compensatory movements; it does not disturb the equilibrium or the righting reaction, nor is the muscle tonus affected to any noticeable degree.

Removal of Both Ampullæ and Otoliths.

I may say at once that in accordance with the findings stated in the last section the presence or absence of the large otolith of the saccule is without influence on the equilibrium reactions and it may be disregarded. If, however, the utricular otolith has been successfully removed and the condition described in a previous section has been attained, namely the retention of compensatory movements to rotations in all planes, the righting reaction, and the maintenance of equilibrium, the consequent removal of the six ampullæ produces at once a profound alteration. The condition of a dogfish deprived of the utricular otolith and the six ampullæ may be stated in the following way. (1) No compensatory movements are made on rotation around any axis whatever. This statement may be modified by saying that in some cases a slow and slight tendency to compensation, requiring many seconds or even minutes for its completion, may be seen. No one familiar with the reactions of the animal would ever confuse this with a labyrinthine reflex. (2) The animal shows no tendency to maintain bodily equilibrium; it swims indifferently back or belly upward. A weak specimen may also come to rest on its

side or back, but a vigorous specimen usually rights itself on the bottom of the tank. In other words the geotropic reactions of the animal are definitely and completely lost; the stereotropic reaction is retained.

CONCLUSION.

The results of my experiments show that the assumption of a sharp differentiation of function between the otolith-bearing, vestibular portions of the labyrinth and the semicircular canals is not justified by the facts. Between the effects of extirpation of the one and of the other set of structures there is more resemblance than contrast. They certainly reenforce each other, for the reactions produced by either one alone are always slower and less vigorous than when both sets of organs are intact. It would not, however, be safe to affirm that the functions are identical. In one respect a difference is apparent; namely, in the response to rotation in a horizontal plane. If the ampullæ are uninjured, compensatory movements occur when the animal is rotated around its dorsoventral axis. I have never seen this reaction in the absence of the ampullæ of the horizontal canals.

SUMMARY.

1. A dogfish from which all six ampullæ have been removed maintains its equilibrium; the righting reactions occur promptly; compensatory movements of the eyes occur in response to rotations in all planes except the horizontal; the compensatory position of the eyes is retained if the animal is held in an abnormal position. Both the static and dynamic functions of equilibrium continue, therefore, after complete removal of all the semicircular canals and all the ampullæ.

2. After complete removal of the otoliths from the vestibules without injury to the ampullæ the animal maintains its equilibrium in the water, rights itself promptly, and makes compensatory motions to rotations in all planes. If held in an abnormal position the compensatory position of the eyes is maintained. Both static and dynamic functions of equilibrium continue.

3. Destruction of both the semicircular canals and the otolith organs completely abolishes all compensatory movements and equilibrium reactions of labyrinthine origin.

4. It is pointed out that these observations do not justify the theory of Mach and Breuer that the ampullæ and semicircular canals are the organs for the dynamic functions of equilibrium, and that the otoliths are the organs for the static functions of equilibrium.

• 5. The new experiments recorded in this paper show that the ampullæ alone (without the otoliths) suffice for all the dynamic and all the static functions of equilibrium of the ear; and that the otolith organs (without the ampullæ) suffice for all the static and for all the dynamic functions of equilibrium of the ear with the exception of the response to a rotation of the animal in a horizontal plane.

STUDIES ON BIOLUMINESCENCE.

X. CARBON DIOXIDE PRODUCTION DURING LUMINESCENCE OF CYPRIDINA LUCIFERIN.

By E. NEWTON HARVEY.

(From the Physiological Laboratory, Princeton University, Princeton.)

(Received for publication, September 5, 1919.)

All luminous animals require oxygen for luminescence but it is not known whether they give off CO_2 as a result of the luminescence. Luminous bacteria produce carbon dioxide but this is no doubt the carbon dioxide of respiration. Even if we could prove that luminous bacteria produced more carbon dioxide than certain non-luminous bacteria, this would not necessarily mean that the excess carbon dioxide was produced during luminescence. Different organisms produce very different quantities of CO_2 per body weight and in no necessary relation to luminescence. In 1855, Fabre¹ published results which indicated that non-luminous portions of a mushroom, *Agaricus olearius*, produced less CO_2 (2.88 cc. of CO_2 per gm. in 36 hours at $12^\circ\text{C}.$) than luminous parts (4.41 cc. of CO_2 per gm. in 36 hours at $12^\circ\text{C}.$). There are, however, many reasons besides luminescence why one part of a plant might produce more CO_2 than another.

There are also many facts which indicate that luminescence is in no way connected with the respiration of cells. This is quite obvious in the case of animal cells where respiration is continuous and luminescence appears only as the result of stimulation of the cell. But even in bacteria where respiration and luminescence are both continuous, the two processes are distinct. Some luminous bacteria are facultative anaerobes and will multiply and respire but will not luminesce in the absence of oxygen.² McKenney³ found that *Bacillus phosphorescens* will grow rapidly in 0.5 per cent ether without pro-

¹ Fabre, J. H., *Am. Sc. Nat.*, 1855, iv, 179.

² Beÿerinck, M. W., *Arch. Neer.*, 1889, xxiii, 416.

³ McKenney, R. E. B., *Proc. Biol. Soc. Washington*, 1902, xv, 213.

ducing light. If the temperature of a luminous bacterium is gradually raised, respiration increases continuously up to a relatively high maximum, whereas the luminescence decreases rapidly above a relatively low optimum. The two processes are not equally affected by increasing temperature.

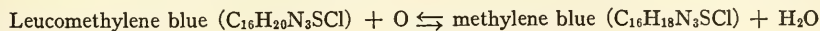
It is obvious that, to measure carbon dioxide production during luminescence, we must use cell-free solutions of the oxidizable material of luminous animals, luciferin, and oxidize it suddenly in order to have the maximum amount of CO_2 produced at one time. This experiment can be carried out with the luciferin of *Cypridina hilgendorffi*, an ostracod crustacean. A brilliant luminescence results from adding a small amount of luciferase solution to a solution of *Cypridina* luciferin. The preparation of these solutions has been described in a previous paper.⁴

Carbon dioxide production was tested by determining if any change in acidity, which might come from the CO_2 produced, occurs when solutions of luciferin and luciferase are mixed. After several attempts to measure acidity by adding an indicator (thymolsulfonephthalein) to the solution, this method was given up because the luciferin and luciferase solutions are yellowish in color, which interferes with the yellow-blue color change of the thymolsulfonephthalein. The electrometric determination with the hydrogen and 0.1 N KCl calomel electrode is the most sensitive. A McClendon electrode and Leeds and Northrup potentiometer were used. The acidity of the luciferin solution, luciferase solution, and the two after mixing was found to be the same, $\text{pH}=9.04$. Therefore, not enough CO_2 is produced to affect the hydrogen ion concentration.

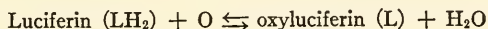
As both luciferin and luciferase solutions contain proteins and as luciferase is certainly and luciferin probably a protein, it will be seen that their buffer value is relatively high. The luciferin and luciferase solutions, although prepared with distilled water, no doubt contain also a small amount of buffer salts. Our experiments show this much, however, that not enough CO_2 is produced during luminescence to saturate the proteins in solution, including luciferin and luciferase themselves. The reaction responsible for luminescence, the oxida-

⁴ Harvey, E. N., *J. Gen. Physiol.*, 1918-19, i, 269.

tion of luciferin, is, therefore, not to be compared to the reactions in cells giving rise to the carbon dioxide of respiration. As I have previously suggested⁵ it is probably of a type similar to the oxidation of a leuco dye to the dye itself. In the case of methylene blue this reaction may be represented as follows:



In the case of luciferin we may have



Whether the oxidation of luciferin really proceeds with the formation of H_2O or whether it involves the actual addition of oxygen, as in the oxidation of hemoglobin, is uncertain. Like methylene blue and oxyhemoglobin the oxyluciferin can be again reduced⁵ but the reduction resembles that of methylene blue in that it will not take place by the mere removal of oxygen, as will the reduction of oxyhemoglobin, but requires also the addition of some reducing agent.

The fact that the reaction luciferin-oxyluciferin is reversible is in itself evidence that no carbon dioxide is produced. Reactions resulting in CO_2 production involve considerable energy changes (heat production) and are reversed only with the greatest difficulty and with the addition of a large amount of energy. Whether any considerable amount of heat is produced during oxidation of luciferin will be discussed in the following paper.

⁵ Harvey, E. N., *J. Gen. Physiol.*, 1918-19, i, 133.

STUDIES ON BIOLUMINESCENCE.

XI. HEAT PRODUCTION DURING LUMINESCENCE OF CYPRIDINA LUCIFERIN.

By E. NEWTON HARVEY.

(From the Nela Research Laboratory, National Lamp Works of the General Electric Company, Cleveland.)

(Received for publication, September 5, 1919.)

Dubois¹ obtained some evidence of infra-red radiation given off during the flash of the West Indian firefly, *Pyrophorus*. Langley and Very² also at first observed a slight heating of their bolometer from the light of the same insect, but in a later investigation, Langley³ was unable to detect any heating of his bolometer whatsoever. Coblentz,⁴ using a vacuum thermopyle, could detect no infra-red radiation from the American firefly, *Photinus*. Ives⁵ also, using a phosphor-photographic method, found no infra-red radiation in the light of *Photinus*. We must attribute the earlier evidence to experimental errors and conclude that the light of luminous animals contains no rays of wave-length longer than the visible.

This does not mean, however, that no heat is produced by the reaction which produces the luminescence. A temperature change of a few thousandths or hundredths of a degree would evolve no measurable radiation. Coblentz⁴ first studied the problem of heat production in the firefly, using a thermo-couple as the measuring instrument. He came to the conclusion that the temperature of the insect was slightly lower than the temperature of the air and that the luminous segments were slightly hotter than the non-lumi-

¹ Dubois, R., *Bull. Soc. Zool. France*, 1886, xi, 1.

² Langley, S. P., and Very, F. W., *Am. J. Sc.*, 1890, xl, series 3, 97.

³ Langley, S. P., *Am. Astrophys. Obs.*, 1902, ii, 5.

⁴ Coblentz, W. W., *Carnegie Institution of Washington, Publication No. 164*, 1912.

⁵ Ives, H. E., *Phys. Rev.*, 1910, xxxi, 637.

nous segments. No definite increase or decrease in temperature could be established during the flash of the firefly. However, further work on the firefly is much to be desired.

The use of a living animal for such measurements introduces a possible source of error, in that any contraction of the muscles of the animal will produce heat which may add to an increase, or mask a decrease of temperature during luminescence. Utilization of extracts of luminous animals avoids the complications due to muscular contraction. From *Cypridina hilgendorffii*, a small crustacean, may be prepared a solution of luciferin, an oxidizable substance, and a solution of luciferase, a catalyzer which accelerates the oxidation of luciferin with light production. By bringing the solutions of luciferin and luciferase to the same temperature and then mixing them, one can measure any increase or decrease of temperature which occurs during the luminescence resulting from mixing. We can thus gain some idea of the heat of oxidation of luciferin.

Although the experiment sounds very simple it is actually somewhat difficult to carry out. The attainment of temperature equilibrium between two solutions is very slow when one wishes to obtain it to within 0.001°C. of the same temperature. After many attempts, the following arrangement of apparatus (Fig. 1) was found most satisfactory. About 10 cc. of luciferin solution were placed in the inner tube (D) of a special non-silvered thermos bottle (A). About 1 cc. of luciferase solution was placed in a very thin-walled glass tube (E) which was immersed in the luciferase solution and connected with a small motor so that it could be slowly but constantly rotated, thus stirring the solutions. Thermo-couples (L and M) of advance (0.008 inch) copper (No. 30, B and S enamel-insulated) wire were parafined and placed in each tube and the copper wires connected through a copper double throw switch (C) with a Leeds and Northrup d'Arsonval wall galvanometer (No. 34,637, silver strip suspension) of 35 ohms resistance and 310 megohms sensitivity. The constant temperature junctions (N) were placed in a large Dewar flask (B) and filled with water at approximately the same temperature as the luciferin solution. 1 mm. galvanometer scale division represented 0.003°C. and the division readings could be estimated to tenths. By means of a glass rod (F) placed in the tube containing luciferase solution,

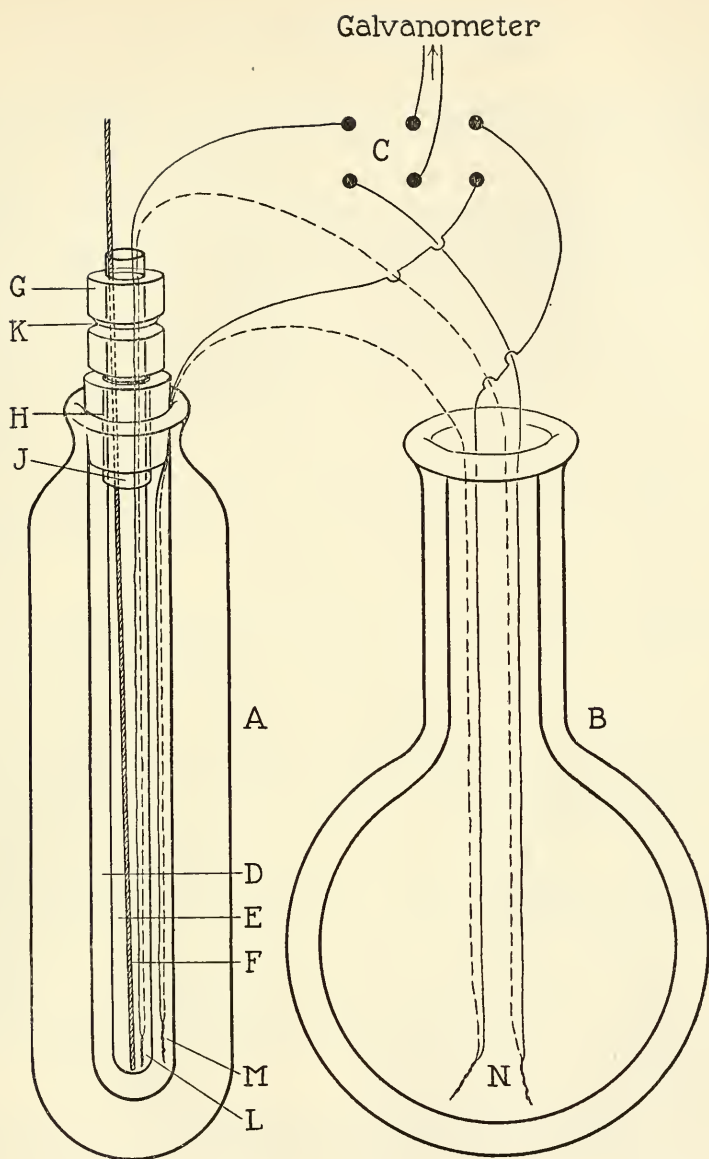


FIG. 1. Apparatus for determining heat production during luminescence of luciferin. A, special thermos tube. B, Dewar flask for constant temperature junctions. C, copper double throw switch. D, tube containing luciferin solution. E, tube containing luciferase solution. F, glass rod for breaking E. G, rubber stopper with groove, K, for pulley cord. H, cork closing thermos tube. J, brass sleeve in H allowing rotation of E. L, thermo-couple in luciferase solution. M, thermo-couple in luciferin solution. N, constant temperature junctions.

this tube could be broken and the luciferase and luciferin solutions mixed.

It was found that even after the luciferase and luciferin solutions came to the same temperature within the thermos bottle, this was not necessarily the same as that of the room, and a slow rise or fall occurred as indicated by a slow drift of the galvanometer coil. Readings of each thermo-couple on the galvanometer scale were therefore taken at 1 minute intervals for some time before and after mixing the luciferin and luciferase solutions and plotted as curves. Control experiments were also carried out in exactly the same manner as the luciferin-luciferase experiments but water was placed in the two tubes instead of luciferin and luciferase. Figs. 2 and 3 give typical experiments with water and with luminescent solutions respectively. As can be seen from the curves, the rise in temperature in each case figured is 0.006°C. , or two scale divisions.

With both control (water) and luciferin experiments there was a slight rise in temperature on mixing the liquids in the two tubes. The average rise of five control (water) experiments was 0.0054°C. and the average rise of five luciferin experiments was 0.0048°C.

In one control experiment there was no change in temperature on mixing and in one luciferin experiment there was a slight drop in temperature (0.0045°C.) on mixing. The average rise in temperature is no doubt due to heat from friction in the mixing of the liquids and the breaking of the glass tube. The difference in the average rise of control and of luciferin experiments is so small (0.0006°C.) as to have little significance. We may therefore conclude that if any temperature change occurs during the luminescent reaction it is certainly less than 0.001°C. and probably less than 0.0005°C. ; too small to be measured by this method.

To prepare the luciferin solution, 2 gm. of dried *Cypridina* were dissolved in 20 cc. of hot water and 10 cc. of this 10 per cent solution were used in the thermos bottle in the above experiments. If we assume that 1 per cent of the dried *Cypridina* is luciferin, 0.1 gm. of luciferin on oxidation was not able to change the temperature of the 10 cc. (in reality 11 cc., since 1 cc. of luciferase solution was mixed with the 10 cc. of luciferin) of solution 0.001°C. This means that 1 gm. of luciferin liberates less than 0.1 calorie during the luminescence accompanying oxidation.

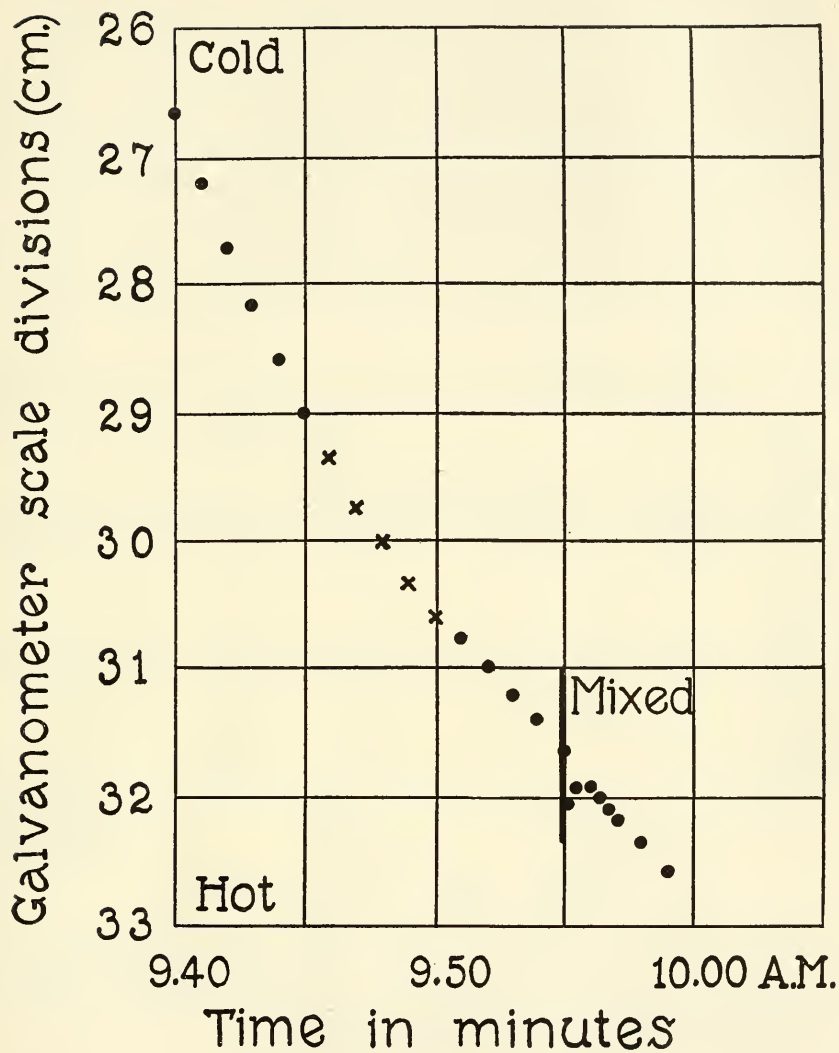


FIG. 2. Curve showing temperature change when two tubes containing water at the same temperature are mixed. 0.1 galvanometer scale division = 0.003°C . Dots represent readings of thermo-couple in tube D; crosses, readings of thermo-couple in tube E.

Since 1 gm. of glucose liberates 4,000 calories on complete oxidation to CO_2 and H_2O , it will be seen that the oxidation of luciferin is a very different type of reaction from the oxidation of glucose. As I have already suggested it is similar to the oxidation of reduced hemoglobin or the oxidation of leucomethylene blue to methylene blue. According to Barcroft and Hill⁶ 1.85 calories are produced per gm. of hemoglobin oxidized. I have been unable to find figures for the

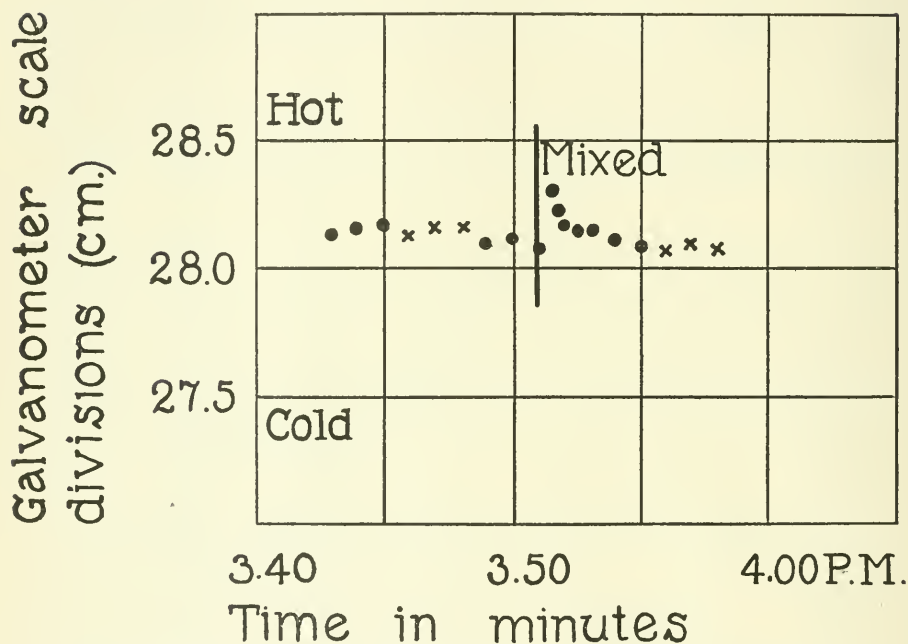


FIG. 3. Curve showing temperature change when luciferin and luciferase solutions at the same temperature are mixed. 0.1 galvanometer scale division = 0.003°C . Dots represent readings of thermo-couple in luciferin solution; crosses, readings of thermo-couple in luciferase solution.

heat exchange during oxidation of leuco dyes, but it is no doubt also small.

The production of carbon dioxide always involves the evolution of considerable heat. Since luciferin evolves no measurable amount

⁶ Barcroft, M. A., and Hill, A. V., *J. Physiol.*, 1909-10, xxxix, 411.

of heat on oxidation we have further evidence in support of that presented in a previous paper, that no carbon dioxide is produced during luminescence of luciferin. The energy change involved is very small indeed. It is on first thought surprising that so bright a luminescence as that of *Cypridina* should result from a reaction involving only a very small amount of heat. If we recall, however, that the eye is an extraordinarily sensitive instrument (responding to 10^{-9} ergs per second), which can detect light so weak, that, if it were converted into heat, would take 60,000,000 years to raise 1 gm. of water $1^{\circ}\text{C}.$, we may realize that, after all, a very small heat production during oxidation of luciferin may be sufficiently great to account for its luminescence.

It gives me pleasure to acknowledge the kindly interest and assistance in setting up apparatus of Dr. W. Weniger of the Nela Research Laboratory.

THE ISOELECTRIC POINTS OF THE PROTEINS IN CERTAIN VEGETABLE JUICES.

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(Received for publication, August 21, 1919.)

The preparation in the dry condition of systems rich in protein has assumed new importance in the practise of dehydrating vegetables and meats. Until recently the removal of water and the preservation of the original appearance of the foodstuffs have been considered the most important criteria for success in the operation. But acceptability of the dehydrated product has varied largely. In certain instances vegetables have lost important nutritional qualities upon dehydration or shortly thereafter. Others have been slow to reabsorb water. Occasionally dehydrated material has spoiled.

Information regarding the nature of the proteins in such systems should explain, in part, the causes of variation, set up criteria for change upon dehydration, and lead gradually to the perfection of processes.

With this in view the characteristics of the proteins in the juices of the potato, of the carrot, and of the tomato have been studied. Their isoelectric points and their solubility at different hydrogen ion concentrations have been determined. The acidity of the juices of these vegetables together with this information suggests the state in which the proteins exist in nature.

The Significance of the Isoelectric Point.

The classification of simple proteins depends upon their solubility in water containing different concentrations of inorganic salts (albumins from globulins), different concentrations of hydrogen ions (albumins and globulins from glutelins), or different concentrations of alcohol

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(prolamines).¹ But within each group state is affected by change in concentration of these factors. Thus the solubility of an albumin, a globulin, or a glutelin is variable with the amount of alcohol contained in the solvent; and the solubility of an albumin or a globulin is variable with the hydrogen ion concentration, and with the nature and amount of the dissolved electrolytes.

Recent investigations have suggested the course of certain of the chemical reactions that involve changes in the solubility of proteins. It appears that, whatever the superimposed complexity resulting from the colloidal nature of the systems, the behavior of protein substances, whether simple or conjugated, is dependent in large part upon their ionization as amphoteric electrolytes.^{2,3,4} As amphoteric electrolytes, proteins combine with either acids or bases, but at a particular hydrogen ion concentration they exist most nearly uncombined. The value of this singular point in ionization and in behavior is characteristic of each protein and is termed its isoelectric point. The isoelectric point is thus a measure of the relative strength of protein as acid and base.

The combination of the protein molecule with acid or with basic radicles effects a change in its solubility and in its hydration. The compounds with simple acids or bases vary greatly in their ability to absorb water and to dissolve in water, but in the neighborhood of the isoelectric point protein substances are usually less soluble and less swollen than elsewhere. Empirical evidence for this conclusion antedates the theory for this phenomenon. Both in the laboratory and in industry the preparation of proteins has depended largely upon their lesser solubility near the isoelectric point.

The Determination of the Isoelectric Point.

The migration of charged particles in an electric field is termed cataphoresis. The charge of a protein depends upon its ionization.

¹ Recommendations of the Committee on Protein Nomenclature, *Am. J. Physiol.*, 1908, xxi, p. xxvii.

² Hardy, W. B., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 413. Sørensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1917, xii.

³ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 237.

⁴ Henderson, L. J., Cohn, E. J., Cathcart, P. H., Wachman, J. D., and Fenn, W. O., *J. Gen. Physiol.*, 1918-19, i, 459.

Proteins can ionize as acids or as bases. As acids they migrate to the anode, as bases to the cathode. The nature of the ionization of the protein can thus be inferred from the direction of its migration. When it does not migrate in either direction the protein exists at its isoelectric point.

The isoelectric point of certain vegetable proteins was determined by the method of cataphoresis. The juice of the vegetable was placed in a U-tube, between electrodes charged with a potential difference, and the direction of the protein migration determined. The migration of protein during cataphoresis was followed both by determining nitrogen in the arms of the U-tube and by heating the liquid from the arms and noting in it the appearance of coagulated protein. The technique described by Coehn⁵ and by Michaelis⁶ was so modified as to meet the needs of the present research.

Unless several precautions are observed in investigating the direction and amount of the migration of protein compounds in an electric field the results are scarcely interpretable. The dipping of electrodes into the protein solution especially complicates the phenomenon⁷ since proteins are either precipitated or rendered more soluble at the electrodes.⁸ This is brought about, in part, by the accumulation of H^+ and OH^- ions at the cathode and anode respectively. Landsteiner and Pauli obviated these disturbances by using a three chambered vessel in determining the isoelectric point of very pure egg albumin.⁹ Each chamber contained the protein solution, and migration was detected by determining nitrogen in each after the passage of current. Instead of protein solutions in the end-chambers Michaelis substituted a buffer solution of the same hydrogen ion concentration.

The particular form of apparatus devised for use in these experiments is represented in Fig. 1.¹⁰ The protein solution was placed below and between the stop-cocks, BB, of a U-tube and above was

⁵ Coehn, A., *Z. Electrochem.*, 1909, xv, 652.

⁶ Michaelis, L., *Biochem. Z.*, 1909, xvi, 81.

⁷ Haas, A. R. C., *J. Phys. Chem.*, 1918, xxii, 520.

⁸ Robertson, T. B., *J. Phys. Chem.*, 1911, xv, 179.

⁹ Landsteiner, K., and Pauli, W., *Verhandl. Kong. inn. Med.*, 1908, xxv, 571.

¹⁰ The dimensions of the apparatus are of importance only in calculating movement of protein from the nitrogen concentration before and after cataphoresis. The volume of liquid contained in each arm was 7.4 cc. and in the central chamber below the arms (including stop-cocks) 13.4 cc.

placed a buffer solution of the same hydrogen ion concentration extending to stop-cocks, AA, and conveniently introduced at DD by creating a slight vacuum at C. Between the buffer solution (at A) and the zinc sulfate in the non-polarizable electrodes a sodium chloride solution was placed to avoid the formation of insoluble zinc salts. It was ascertained that the sodium chloride never met the protein by testing for the faster moving chlorine ion in a drop of solution pipetted from the arms of the U-tube. Once filled the level of the solutions was adjusted through C. The stop-cocks BB were then opened and, provided there was no diffusion, a 110 volt direct

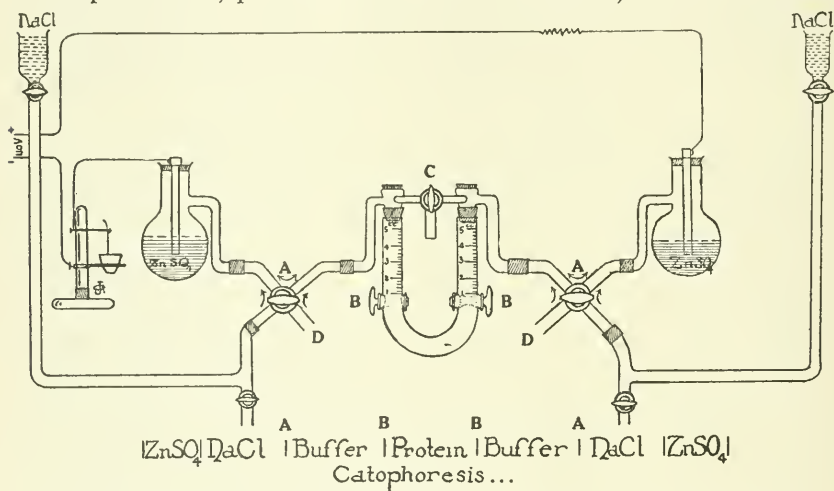


FIG. 1. Apparatus for the determination of protein migration in an electric field.

current was passed in series through the apparatus and a silver coulometer. The deposit of silver in the latter recorded the flow of current during cataphoresis. The drop in voltage across BB was approximately 3 volts, but varied in different experiments with the conductivity of the protein solution. Cataphoresis was carried on in a thermostat at $25^\circ \pm 1^\circ\text{C}$.

The Protein of the Potato.

About 8 per cent of the solids of the potato is protein and about 4 per cent is ash.¹¹ As a result, more than half the protein is dis-

¹¹ The average of all the values given in König, J., *Chemie der menschlichen Nahrungs-und Genussmittel*, Berlin, 4th edition, 1903, i, 713.

solved by the high concentration of electrolytes in the juice that can be squeezed from the potato. With the exception of a small amount of proteose only one well defined protein, the globulin tuberin,¹² has been isolated from the potato. It is present to the extent of from about 1 to 2 per cent in the juice, and was isolated and prepared from this and from the sodium chloride extract of the whole potato.

The Hydrogen Ion Concentration of Potato Juice.—The hydrogen ion concentration of the juice of the potato was slightly less than $10^{-6}N$. When the juice was freed from suspended material—mostly starch—by filtration through pulp, the apparent hydrogen ion concentration was further reduced to about $10^{-7}N$. It was then perfectly clear but darkened by the action of an oxidase intimately related to the globulin in location and in behavior. The oxidase was more active the lower the hydrogen ion concentration. A study of the action of the oxidase has been reported from this laboratory.¹³

The Precipitation of Tuberin—A precipitate separated upon the addition to the juice of the potato of either acid or alkali. The alkaline precipitate was less voluminous, more gelatinous, and more variable in amount than the acid precipitate. It was at first soluble in excess alkali, but later was denatured. Upon the addition of acid to potato juice a white, flocculent precipitate¹⁴ appeared at about pH 6. The precipitate increased in amount and then gradually redissolved upon the addition of more acid. At acidities greater than those reported a protein precipitate gradually reappeared. This precipitate increased with increase in acidity and time and did not resemble a globulin in behavior.

The volumes of the precipitates that settled when different amounts of acid were added to potato juice in cylindrical vessels are recorded in Table I. In every instance the greatest precipitation appeared at a hydrogen ion concentration near $10^{-4}N$. In bulk, however, and rate of settling, the precipitates varied to an extent which could not

¹² Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1896, xviii, 575.

¹³ Falk, K. G., McGuire, G., and Blount, E., *J. Biol. Chem.*, 1919, xxxviii, 229.

¹⁴ Upon standing, the protein frequently separated as a result of acid produced by bacteria.

be accounted for by differences in procedure. This variation was no greater than the variation in the amount of tuberin in the juice, but neither the amount of the precipitate nor its volume was dependent merely upon the concentration of protein. More probably it was related to differences in the content of electrolytes in the juice, for the addition of sodium chloride had a great effect upon the behavior both of the acid and of the alkaline precipitate.

TABLE I.
Measurements on Potato Juice.

N HCl added to 100 cc. of potato juice.	Gm. of nitrogen in potato juice.														
	0.213	0.238	0.242	0.292	0.309	0.310	0.213	0.238	0.286	0.315	0.213	0.238	0.242	0.309	0.310
	Volume of protein precipitate.						Nitrogen in filtrate from precipitate.				pH of filtrate from precipitate.				
	cc.	cc.	cc.	cc.	cc.	cc.	gm.	gm.	gm.	gm.					
16		0						0.225							
15				0	0	1			0.275					2.18	2.30
13	9						0.210				2.90				
12						7									2.74
10	25			7	17		0.178		0.242		3.15			2.95	
8		12	80	22	54	20		0.182	0.243			2.75	3.45	3.35	3.57
7	84						0.179				3.60				
6			93	61	64				0.239	0.240			3.85	3.84	
5	88		96		68	23	0.187		0.251	0.242	4.27		4.27	4.12	4.32
4		30	91	30	82			0.200	0.251	0.248		4.07		4.39	
3	92				56	17	0.204		0.251	0.249	4.80			4.73	4.93
2		18			18			0.199		0.255		4.60		5.35	
1		4			0	0		0.220				5.45		6.31	6.15
0	0	0		0	0	0	0.213	0.238	0.286	0.315		6.38		7.00	6.80
N NaOH															
1		4			12	6				0.291		7.65		7.78	7.55
2		9			15	6				0.290		8.66		8.22	8.19
4		5			16	6				0.293		9.50		8.84	8.89
6		3			18	7				0.301				9.39	9.35
8					34									9.78	

This is illustrated by the experiment recorded in Table II in which different amounts of sodium chloride were added to juice to which the same amount of hydrochloric acid had been added. The rates of settling and the volumes of the resulting precipitates were observed

and the nitrogen content of the juices was determined after the precipitate had been removed by centrifuging. Small amounts of sodium chloride greatly decreased the volume of the precipitate and also increased the solubility of the tuberin, though not to the same extent. The effect of sodium chloride upon the solubility of tuberin and upon the apparent hydrogen potential of systems containing it is different not only in amount but in kind at different hydrogen ion concentrations. The relation between this change and the iso-electric point will be considered in another report.

TABLE II.
Effect of Sodium Chloride upon the Precipitation of Tuberin by Acid.

NaCl added to 100 cc. of potato juice + 5 cc. of N HCl.	Volume of precipitate.	Nitrogen in filtrate.
gm.	cc.	gm.
0	97	0.192
0.5	98	0.195
1.0	21	0.204
2.0	20	0.199
4.0	29	0.196
8.0	61	0.178

The Solubility of Tuberin.—The solubility of tuberin at different hydrogen ion concentrations was estimated by determining the nitrogen content of potato juice to which sodium hydroxide or hydrochloric acid had been added in different amounts and from which any resulting precipitate had been removed by filtering or centrifuging. The results of four different experiments, in two of which the volumes of the precipitate were also determined, are reported in Table I. In each tuberin was least soluble in the juice of the potato at hydrogen ion concentrations slightly greater than 10^{-4}N , where precipitation occurred. Precipitation also occurred at hydrogen ion concentrations near 10^{-8}N . The amount of these precipitates was very different, but neither the addition of acid nor of alkali completely precipitated the protein in potato juice. Indeed no more than 25 per cent of the total nitrogen in the juice of the potato was ever found in the most copious acid precipitate nor more than 8 per cent in the largest alkaline precipitate. A curve representing the solubility of tuberin in potato juice thus passes through two minima and a maximum, the latter at the reaction of potato juice (Fig. 2).

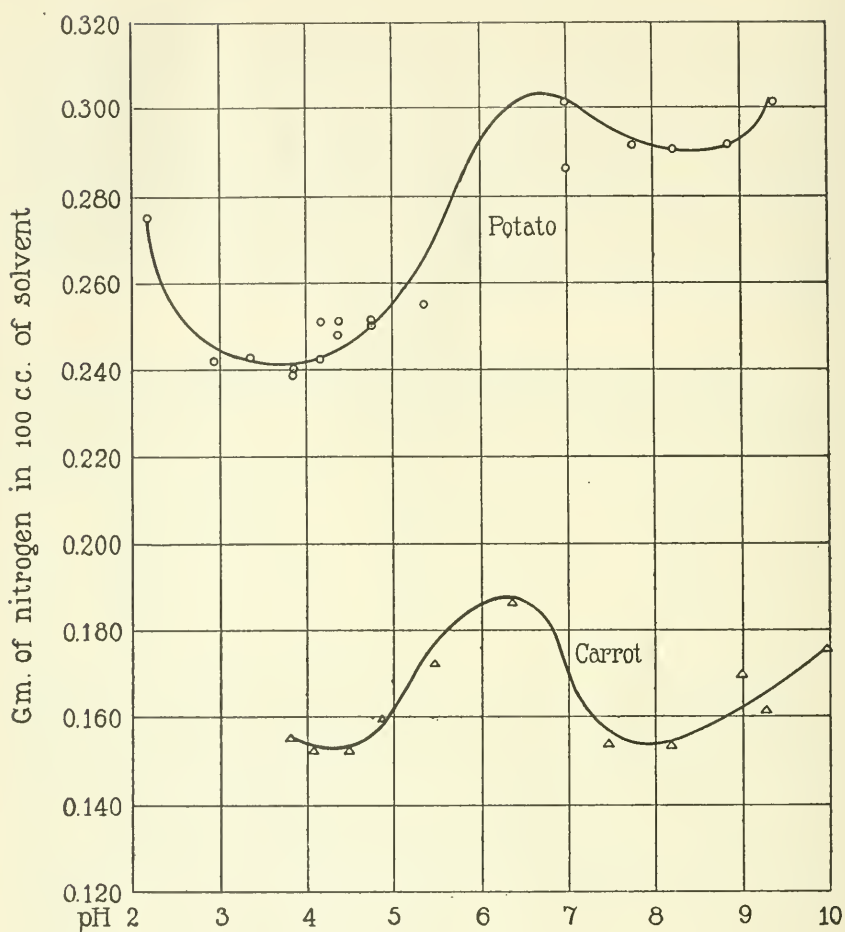


FIG. 2. Solubility of protein in vegetable juices at different hydrogen ion concentrations.

The Compounds of Tuberin.—The changes in the solubility of tuberin that have been described resulted, in part, from the combination with acids and bases of the basic and acid radicles in the juice of the potato. The amount of such combination is calculable if the hydrogen ion concentration is determined. It is equal to the difference between the measured hydrogen ion concentration and

that which would have resulted from the added acid or alkali had there been no combination. It is indicated by the changing slope of the curve representing the titration if the amount of normal acid or alkali is represented as ordinate and the hydrogen ion concentration as abscissæ (Fig. 3).

The curve representing the titration of potato juice with acid and alkali passes through a point of inflection at a hydrogen ion

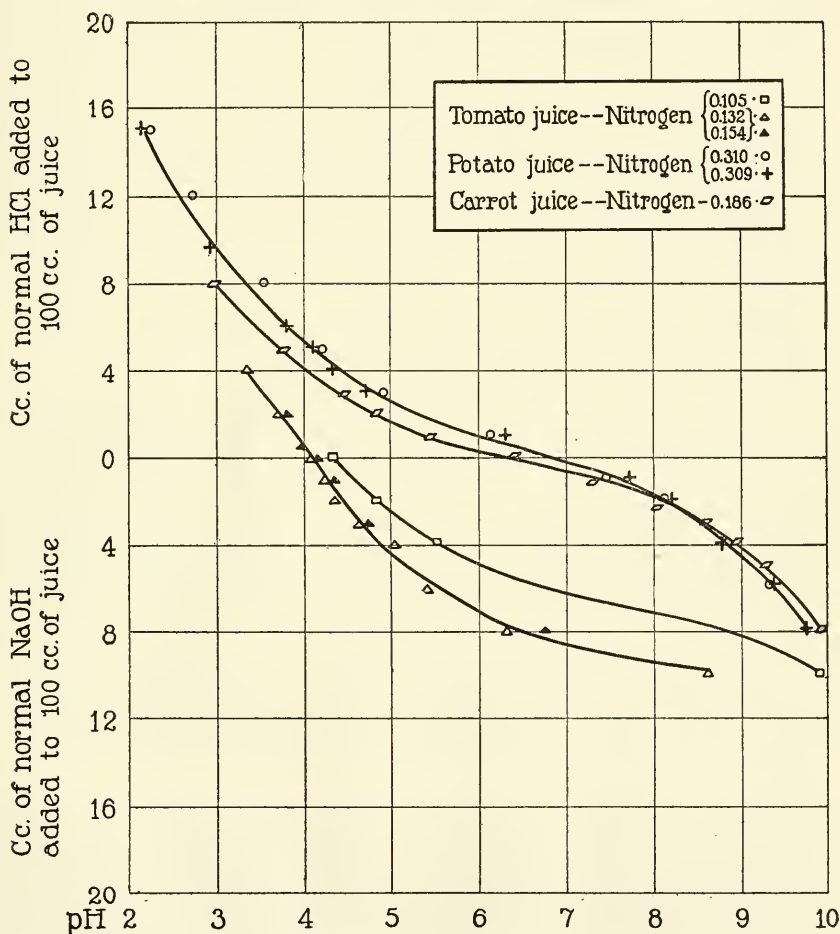


FIG. 3. Titration of vegetable juices.

concentration near the second ionization constant of phosphoric acid ($1.6 \times 10^{-7} = \text{pH } 6.8$). But the strong buffer action, indicated by the steepness of the titration curve, throughout the range investigated cannot be attributed merely to the presence of phosphoric acid nor to the other organic and inorganic weak acids that chemical analyses reveal. The increase in steepness of the titration curve in the range, acid to pH 4.5 on the one hand and alkaline to pH 8.5 on the other, is largely due to the dissociation of the protein compounds that exist in the potato and their recombination with strong acids and bases with the retention of hydrogen and hydroxyl ions. The formation of new compounds at these particular reactions is suggested by a comparison of the titration curve (Fig. 3) with the curve representing the solubility of tuberin in the juice of the potato at different hydrogen ion concentrations (Fig. 2). Each change in solubility is seen to affect the slope of the titration curve, but in different ways. Thus, nearly three times as much acid was required to redissolve tuberin as to precipitate it. Osborne has made a similar observation on another globulin, edestin.¹⁵

The Isoelectric Point of Tuberin.—In an electric field the protein in the juice of the potato migrated toward the anode. It bore, therefore, a negative charge. No change followed an increase in the alkalinity of the juice. The direction of migration of the protein was, however, reversed by the addition of acid. In Table III are collected the results of many experiments. In all the direction of migration of the protein changed at a slightly lower hydrogen ion concentration than 10^{-4}N . At that acidity the protein did not migrate in either direction. It existed at its isoelectric point. At acidities greater than the isoelectric point protein migrated to the cathode. Under these circumstances tuberin apparently ionized as a base, and dissolved as an acid compound.

A second change in direction of the migration of protein in very acid juice occasionally occurred in our early experiments. This change was probably apparent and must be explained as relative to the increased passage of water at these acidities, since it was later averted by increasing the buffer in the arms of the cataphoresis apparatus.

¹⁵ Osborne, T. B., *J. Am. Chem. Soc.*, 1902, xxiv, 39.

The flow of current during cataphoresis was measured, as has been said, by a silver coulometer in series with the apparatus. Since the juice of the potato contains both free electrolytes and electrolytes combined with tuberin, deductions regarding the transference of current by tuberin cannot readily be drawn from these data (Table III). It would be reasonable to suppose, however, that as the acidity of the solutions increased their specific conductivity would be increased

TABLE III.
Migration of Protein in Potato Juice during Cataphoresis.

N HCl added to 100 cc. of potato juice.	Amount of nitrogen in 5 cc. of filtrate.	pH of filtrate.	Conditions of cataphoresis.			Protein migration during cataphoresis.			
			Buffer solution used (M/20).		Flow of current.	Direction.	Amount of nitrogen after cataphoresis in 5 cc.		
			C ₂ H ₄ O ₂	C ₂ H ₃ O ₂ Na	Silver per hr.		Anodal chamber.	Central chamber.	Cathodal chamber.
cc.	mg.		cc.	cc.	gm.		mg.	mg.	mg.
16			*			Cathodic.			
10	9.4	3.10	19.6	0.4	0.0091	"	(0.2	4.7	5.2)‡
8		3.45	19.0	1.0	0.0060	"			
7	8.4	3.60	18.5	1.5	0.0145	"	0.7	8.0	0.8
6		3.85	17.0	3.0	0.0185	"			
5	8.9	4.27	14.0	6.0	0.0338	"	0.5	7.2	0.6
5	11.1	4.27	14.0	6.0	0.0253	"			
4	12.1	4.50	12.0	8.0	0.0050	Anodic.	3.9	9.9	0.3
3		4.80	8.0	12.0	0.0048	"			
0	14.6	6.22	2.0†	8.0		"			

* Citrate mixture used.

† M/60 phosphate mixture used.

‡ Cataphoresis continued for 17½ hours.

because of the high mobility of hydrogen ions. None the less, the flow of current during cataphoresis seems to pass through a maximum at a hydrogen ion concentration slightly less than $10^{-4}N$ (Table III). Presumably conductivity passes through a maximum in the neighborhood of the isoelectric point.

The hydrogen ion concentration which numerically represents the isoelectric point of tuberin reveals the relatively stronger acid proper-

ties of this protein. The amount of acid required to titrate the protein to its isoelectric point is a measure of the amount of combined protein that exists in the juice of the potato and is dissociated by the addition of acid. The soluble salt of this protein that is formed on the other side of its isoelectric point evidently contains three times as much acid as was necessary to dissociate the compound that existed in nature. The relation between this compound and those that exist at other hydrogen ion concentrations and the state in which they exist are suggested by these data.

The Protein of the Carrot.

Remarkably similar in behavior to potato juice is the juice of the carrot. Existing at approximately the same hydrogen ion concentration it combines acid and alkali to precisely the same extent. Fig. 3 illustrates the essential coincidence of their titration curves. Moreover, protein separates from the juice of the carrot upon the addition either of acid or of alkali. The volume of the precipitate and the nitrogen in the filtrate are both recorded in Table IV. As in the potato, the acid precipitate of greatest bulk appeared at a hydrogen ion concentration slightly less than 10^{-4}N . The alkaline precipitate appeared near 10^{-8}N .

The isoelectric point of the protein in juice filtered from the acid precipitate was determined. The change in direction of protein migration also occurred at a hydrogen ion concentration not far from 10^{-4}N . All the measurements upon carrot juice are arranged in tabular form. With one exception—derived from the data upon its solubility—the salient characteristics of carrot protein suggest those of tuberin. The isoelectric point and amphoteric constants of the protein in both vegetables are essentially identical. In nature they exist at approximately the same reaction. A consideration of the curves representing their solubility at different hydrogen ion concentrations suggests that they may exist as somewhat similar compounds (Fig. 2). But whereas in the potato the alkaline precipitate is slight, in the carrot it is almost as great in amount as the acid precipitate.

TABLE IV.
Measurements on Carrot Juice.

N HCl added to 100 cc. of carrot juice.	Gm. of nitrogen in carrot juice.						Direction of protein migration.
	0.186	0.261	0.186	0.186	0.261	0.276	
	Volume of protein precipitate.		Nitrogen in filtrate from precipitate.	pH of filtrate from precipitate.			
cc.	cc.	cc.	gm.				
20		27			1.65		Cathodic.
16		26			2.21	2.10	
12		28			3.07	2.57	
10							
8	13	30		2.98		3.22	Cathodic.
6							
5	15		0.155	3.80			
4	17	36	0.152	4.06	4.37	4.23	Anodic.
3	19		0.152	4.48	4.72		
2	8		0.159	4.86			
1	0		0.172	5.45			Anodic.
0	0	8	0.186	6.43		6.25	"
N NaOH							
1	2		0.153	7.45			
2	5		0.153	8.18			
3	6		0.159	8.67			
4	6		0.169	8.95			
5	8		0.161	9.26			
8	14		0.175	9.92			

The Protein of the Tomato.

The juice of the tomato is very different. A high concentration of organic acids give to the ripe tomato a hydrogen ion concentration of nearly $10^{-4}N$. According to Albahary,¹⁶ the principal acids are malic, phosphoric, and citric. According also to Albahary,¹⁷ the concentration of "albumin" and of "nucleoprotein" decreases during the period of maturation of the tomato and the concentration of free acids increases. As a result of a study of the buffer process in the metabolism of plants, Miss Hempel also suggested that the

¹⁶ Albahary, J.-M., *Compt. rend. Acad. Sc.*, 1908, cxlvi, 336.

¹⁷ Albahary, F.-M., *Compt. rend. Acad. Sc.*, 1908, cxlvii, 146.

extremely high acidity of the ripened lemon might be brought about in some such way.¹⁸

The lack of homogeneity of tomato juice that has merely been squeezed through cheese cloth is manifest. It contains, however, nearly 1 per cent of protein. Filtering the juice removes a large amount of this protein and at the same time appears to decrease the hydrogen ion concentration of the filtrate to nearly $10^{-5}N$. Whatever

TABLE V.
Measurements on Tomato Juice.

N HCl added to 100 cc. of tomato juice.	Gm. of nitrogen in tomato juice.								
	0.105	0.132	0.154	0.105	0.132	0.154	0.105	0.132	0.154
	pH of tomato juice.			Direction of protein migration.			Silver deposited during cataphoresis.		
cc.							gm.	gm.	gm.
4		3.38							
2		3.71	3.80					0.0100	0.0380
1			4.00			Cathodic.			0.0380
0.6						"			0.0160
0	4.35	4.13	4.15	Cathodic.	Cathodic.		0.0609	0.0481	0.0243
N NaOH									
1		4.24	4.32			Cathodic.			0.0481
2	4.85	4.39		Cathodic.			0.0467		
3		4.63	4.79			Anodic.			0.0204
4	5.54	5.05		Anodic.	Anodic.		0.0321	0.0260	
6		5.42							
8		6.35	6.76						
10	9.90	8.68							
20	11.95								

the explanation of the latter observation, the removal of protein by filtration is easily understood. Protein exists largely in suspension.

In entire conformity are the results of cataphoresis (Table V). The unfiltered juice usually migrates to the cathode. A slight reduction in acidity reverses the sign of the protein. The protein, therefore, exists near its isoelectric point and probably slightly on the acid

¹⁸ Hempel, J., *Compt. rend. trav. Lab. Carlsberg*, 1917, xiii.

side of it. In more nearly neutral solution it is soluble and can be freed from other constituents of the tomato by filtering through pulp. It then migrates to the anode. The addition of acid again precipitates the protein at its isoelectric point.

SUMMARY.

The state in which a protein substance exists depends upon the nature of its combination with acids or bases and is changed by change in the protein compound.

The nature of the compound of a protein that exists at any hydrogen ion concentration can be ascertained if the isoelectric point of the protein is known.

Accordingly information regarding the isoelectric points of vegetable proteins is of importance for operations in which it may be desirable to change the state of protein substances, as in the dehydration of vegetables.

The Protein in Potato Juice.—The hydrogen ion concentration of the filtered juice of the potato is in the neighborhood of $10^{-7}N$. Such juice contains the globulin tuberin to the extent of from 1 to 2 per cent.

The character of the compound of tuberin that exists in nature was suggested by its anodic migration in an electric field.

The addition of acid to potato juice dissociated this compound and liberated tuberin at its isoelectric point. The isoelectric point of tuberin coincided with a slightly lower hydrogen ion concentration than $10^{-4}N$. At that reaction it existed most nearly uncombined.

The flow of current during cataphoresis was greatest in the neighborhood of the isoelectric point. This evidence supplements that of the direction of the migration of tuberin, since it also suggests the existence of the greatest number of uncombined ions near this point.

At acidities greater than the isoelectric point tuberin combined with acid. The compound that was formed contained nearly three times as much acid as was needed to dissociate the tuberin compound that existed in nature. At such acidities tuberin migrated to the cathode.

Though never completely precipitated tuberin was least soluble in the juice of the potato in the neighborhood of its isoelectric point.

Both the compounds of tuberin with acids and with bases were more soluble in the juice than was uncombined tuberin.

The nature of the slight precipitate that separated when potato juice was made slightly alkaline was not determined.

The Protein in Carrot Juice.—The isoelectric point of the protein in carrot juice coincided with that of tuberin. Remarkably similar also were the properties of carrot juice and the juice of the potato. Existing in nature at nearly the same reaction they combined with acids and bases to nearly the same extent and showed minima in solubility at the same hydrogen ion concentrations. The greatest difference in behavior concerned the alkaline precipitate which, in the carrot, was nearly as great as the acid precipitate.

The Protein in Tomato Juice.—The protein of the tomato existed in a precipitated form near its isoelectric point. Accordingly it was not present to any extent in filtered tomato juice. If, however, the considerable acidity at which the tomato exists was neutralized the protein dissolved and was filterable. It then migrated to the anode in an electric field. The addition of sufficient acid to make the hydrogen ion concentration slightly greater than $10^{-5}N$ again precipitated the protein at its isoelectric point. At greater acidities migration was cathodic.

IODINE AND THE THYROID.

IV. QUANTITATIVE EXPERIMENTS ON IODINE FEEDING AND METAMORPHOSIS.

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(Received for publication, September 15, 1919.)

This paper deals with experiments undertaken with the object of determining, by quantitative feeding, the approximate amount of elemental iodine necessary to induce complete metamorphosis in normal and thyroidless toad larvæ, reared under identical environmental conditions.

In previous studies on the relation of iodine to the thyroid,¹ as determined by feeding this halogen and its compounds to tadpoles, the experimental evidence led to the suggestion that probably the chief function of the thyroid apparatus, at any rate in the Anura, is the extraction from the blood, and storage of the extremely minute quantities of iodine taken into the body by means of food and water, and the subsequent release of this substance perhaps in a modified form, as the thyroid hormone. The purpose of this experiment was to test the validity of this conclusion, for it is evident, that if the thyroid apparatus in Anura serves the purpose of collecting, storing, and transforming the incoming iodine into a more active hormonal agent, then tadpoles possessing normal thyroid glands should react more promptly by metamorphosis to iodine feeding than tadpoles of similar age and parentage, whose thyroids had been removed before the period of their first functioning, and hence were devoid of the requisite mechanism for collecting, storing, and transforming iodine. Moreover, it was hoped that such an experiment would show whether or not there is an irreducible minimum quantity of iodine required by anurans for metamorphosis.

¹ Swingle, W. W., *J. Exp. Zool.*, 1918-19, xxvii, 397, 417; *J. Gen. Physiol.*, 1918-19, i, 593; *Endocrinology*, 1918, ii, 283.

If this proved to be true, then the minimum figure should be much lower for animals with thyroid glands than for those without.

A brief resumé of the facts brought to light in earlier papers on this subject are: (1) Inorganic iodine and its compounds, iodoform and potassium iodide, greatly accelerate metamorphosis of tadpoles. (2) Anuran larvæ from which the thyroid gland had been removed at its inception (*i.e.* 6 mm. larvæ) and which under normal conditions do not undergo metamorphosis, but grow to abnormal size, quickly transform into frogs when fed elemental iodine. This indicated that iodine is essential for amphibian metamorphosis, that it is the active constituent of the thyroid glands of these animals, and, judging by its physiological effect on thyroidless tadpoles, that it exerts its activity directly upon the cells and tissues of the organisms; *i.e.*, that iodine is capable of functioning like the thyroid hormone in thyroidless tadpoles, and the thyroid-like action is either inherent in the iodine atom, or else the elemental iodine is transformed into a substance similar to thyroid secretion by tissues other than those which normally perform this function. (3) The thyroid follicles of tadpoles on an iodine diet show a greater colloid content than do the glands of normally fed animals. (4) The peculiar action of iodine in accelerating metamorphosis is apparently unique and not possessed by other closely related chemical agents like bromine. (5) Anuran metamorphosis depends upon the amount of iodine secured by the larvæ; the greater the quantity the more rapid the differentiation.

Materials and Observations.

The experimental animals were tadpoles of the common toad, *Bufo lentiginosus*; all came from the same egg mass and hence presumably were of the same parentage. The thyroid anlagen of 100 animals were extirpated at the time of their evagination from the pharyngeal floor, and the larvæ appropriately controlled.² When the larvæ were 9 mm. long, control and thyroidless groups were divided into five cultures each, twenty larvæ to a culture. The containers were large, glass bowls containing 3,000 cc. of tap water. Four thyroidless cultures and four cultures of normal animals were used for experimental purposes; the remaining two cultures served as

²The writer is indebted to Miss M. E. Larson for the larvæ.

checks. The original plan was to use only distilled water, but water distilled by ordinary methods was found toxic for tadpoles. Only quartz distilling was satisfactory. Unfortunately the laboratory facilities for this method were inadequate for producing 30,000 cc. daily, so tap water was substituted. Princeton water possibly contains minute traces of iodine, but if so it is present in such small quantities as to prove negligible in the present experiment.

The nature and amount of the food fed were carefully controlled, and consisted of fat-free casein, yeast, potato starch, and rolled oats. 3 days a week small quantities of *Elodea* were placed in the containers. Toad larvæ do not feed upon the *Elodea* itself, but only upon the diatoms and other organisms living on it. It is evident, from the choice of foods listed, that the possibility that iodine might be present in minute traces was not absolutely ruled out. However, as in the case of the tap water, if present it was insufficient in quantity to affect metamorphosis during the interval the experiment lasted. The water of the cultures was changed daily and the containers were well rinsed. An M/20 stock solution of iodine was employed. The gram concentration of iodine used for the various cultures is given in the following table. This amount was given daily.

Thyroidless cultures.	Concentration of iodine.	Normal cultures.	Concentration of iodine.
Culture 1	0.0000014532	Culture 1	0.0000014532
" 2	0.0000011499	" 2	0.0000011499
" 3	0.0000007666	" 3	0.0000007666
" 4	0.0000003833	" 4	0.0000003833

Frog and toad larvæ are said not to swallow water³ but to absorb it through the integument, consequently this method of iodine administration is very satisfactory. The obvious defect of this method is that it is impossible to determine the exact amount of water absorbed or iodine obtained by the larvæ per day.

For the sake of brevity, all descriptions of the various cultures will be presented in tabular form. Date of first iodine administration, May 19; average total length 9 mm.; no limb buds present. A plus sign indicates that fore limbs have appeared.

³ Boulenger, E. G., Reptiles and batrachians, London and New York, 1914, xiv, 197-199.

Observations of May 26.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	<i>mm.</i>	<i>mm.</i>				
Culture 1.....	15.0	0.80		None.	Good.	None.
" 2.....	16.0	0.65		"	"	"
" 3.....	17.0	0.43		"	"	"
" 4.....	16.5	0.36		"	"	"
Normal iodine-fed.						
Culture 1.....	14.5	2.0 (Toes.)		None.	Thin.	None.
" 2.....	15.5	2.0 (No toes.)		"	"	"
" 3.....	17.0	1.0 " "		"	Good.	"
" 4.....	16.0	0.84		"	"	"
Thyroidless control.....	15.0	0.30		"	"	"
Normal ".....	14.0	0.46		"	"	"

Observations of June 4.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	<i>mm.</i>	<i>mm.</i>				
Culture 1.....	19.5	3.5			Emaciated.	2
" 2.....	20.5	2.5			"	
" 3.....	22.0	1.5			Good.	1
" 4.....	21.0	1.0			"	
Normal iodine-fed.						
Culture 1.....	18.5	5.0			Emaciated.	
" 2.....	19.0	4.0			"	1
" 3.....	21.5	3.0			"	
" 4.....	20.0	1.5			Good.	
Thyroidless control.....	18.5	0.94			"	
Normal ".....	17.5	0.98			"	1

Observations of June 18.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	<i>mm.</i>	<i>mm.</i>				
Culture 1.....	24.5	8.5			Emaciated.	
" 2.....	28.0	8.0			"	
" 3.....	29.0	4.0			Good.	1
" 4.....	27.0	2.5			"	
Normal iodine-fed.						
Culture 1.....	Completed metamorphosis, June 12 to 18.					
" 2.....	21.5	11.0	*	+	Emaciated.	2
" 3.....	23.0	7.5			"	
" 4.....	22.5	5.0			Good.	
Thyroidless control.....	25.0	1.5			"	
Normal ".....	21.5	1.5			"	

* Fore limbs of three animals breaking through the skin; culture metamorphosing.

Observations of June 25.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	<i>mm.</i>	<i>mm.</i>				
Culture 1.....	27.5	10.0	Through.*	+	Thin.	2
" 2.....	30.5	9.5	None.		"	1
" 3.....	32.0	5.5	"		Good.	
" 4.....	29.5	3.0	"		"	
Normal iodine-fed.						
Culture 1.....	Completed metamorphosis, June 12 to 18.					
" 2.....	"	"	"	"	18 " 23.	
" 3.....	24.0	10.5			Thin.	3
" 4.....	24.5	7.0			"	
Thyroidless control.....	28.0	2.0				1
Normal ".....	22.5	3.0				1

* Fore limbs through skin in six animals; culture completed metamorphosis by June 29. Four animals died before resorption of the tail was complete.

It will be noted that Cultures 1 and 2 of the normal iodine-fed larvæ, living in iodine concentrations of 0.0000014532 and 0.0000011499 respectively, completed metamorphosis before the corresponding thyroidless cultures began to transform. It will be shown later that the interval between metamorphosis of normal iodine-fed and thyroidless iodine-fed cultures in equivalent solutions is lengthened the lower the iodine concentration, finally becoming perhaps an indefinite interval in the case of the thyroidless animals.⁴ This strongly indicates that there is an irreducible minimum of iodine concentration for metamorphosis, beyond which thyroidless larvæ are unable to utilize the minute quantities of iodine present, though this may be sufficient to stimulate metamorphosis in animals with intact thyroid apparatus.

⁴ Thyroidless Cultures 3 and 4 unfortunately were lost September 11, owing to neglect to change the water for 52 hours, resulting in decay of the food, especially casein, and consequent death of the larvæ. At this time none of the animals in Culture 4 showed indications of metamorphosis. However, two animals of Culture 3 were about to transform.

Observations of July 5.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	<i>mm.</i>	<i>mm.</i>				
Culture 1.....	Completed metamorphosis, June 25 to 29.					
“ 2.....	33.0	11.5	Through.*	+	Emaciated.	1
“ 3.....	34.5	6.0			“	1
“ 4.....	33.0	4.5				
Normal iodine-fed.						
Culture 1.....	Completed metamorphosis, June 12 to 18.					
“ 2.....	“	“	“	18	“ 23.	
“ 3.....	“	“	“	28	“ July 5.	
“ 4.....	26.5	8.0			Thin.	2
Thyroidless control.....	33.0	3.0				1
Normal “.....	25.0	3.5				

* Fore limbs through skin in three animals of thyroidless Culture 2; culture began metamorphosing July 3 and completed the transformation by July 10.

Observations of July 19.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	<i>mm.</i>	<i>mm.</i>				
Culture 1.....	Completed metamorphosis, June 25 to 29.					
“ 2.....	“	“	“	July 3	“ 10.	
“ 3.....	38.0	6.5			Good.	
“ 4.....	37.0	5.0			“	
Normal iodine-fed.						
Culture 1.....	Completed metamorphosis, June 12 to 18.					
“ 2.....	“	“	“	18	“ 23.	
“ 3.....	“	“	“	28	“ July 5.	
“ 4.....	28.0	10.0			Thin.	
Thyroidless control.....	36.0	4.5			Good.	
Normal “.....	26.0	4.0			“	

Observations of July 30.

Thyroidless iodine-fed.	Total length.	Hind legs.	Fore legs.	Tail atrophy.	Condition.	Mortality.
	<i>mm.</i>	<i>mm.</i>				
Culture 1.....	Completed metamorphosis, June 25 to 29.					
“ 2.....	“	“	“	July 5	“ 10.	
“ 3.....	43.0	7.0			Good.	2
“ 4.....	40.0	6.5			“	1
Normal iodine-fed.						
Culture 1.....	Completed metamorphosis, June 11 to 18.					
“ 2.....	“	“	“	18	“ 23.	
“ 3.....	“	“	“	28	“ July 6.	
“ 4.....	“	“	“	July 27	“ August 2.	
Thyroidless control.....	42.5	5.0			Good.	1
Normal “.....	28.5	5.5			“	1

By July 30, all the normal iodine-fed animals in Cultures 1, 2, and 3 had completed metamorphosis, while in Culture 4 many of the larvæ were transforming, and all had completed the process by August 2. Of the thyroidless animals, Cultures 1 and 2 had metamorphosed earlier, but Cultures 3 and 4 showed on this date no very marked indications of assuming adult characters very soon. These two cultures were fed and tended as usual until September 5. During this interval the animals increased considerably in size, those in Culture 3 averaging 52 mm. with hind legs 10 mm. long; the larvæ of Culture 4 averaged 47 mm. with hind legs 7.5 mm. There were no marked indications of metamorphosis. The two control cultures, one normal and the other thyroidless, averaged in length 27.5 mm. and 40 mm. respectively, with hind legs 7.5 mm. and 5.5 mm. Growth in these two cultures was very slow after July and practically ceased by August 2. The diet appears responsible for this, in part at least. In earlier papers¹ the writer has shown that when fed in minimum quantities iodine stimulates growth in anuran larvæ. This was also found to be the case in the present experiment. The growth rate of frog larvæ reared artificially is so variable, however, the writer lays no great stress upon this point.

The history of thyroidless Cultures 3 and 4 leads to the conclusion that 0.0000007666 and 0.0000003833 concentrations of elemental iodine are insufficient to induce complete metamorphosis in animals devoid of the thyroid apparatus, even when fed over fairly long periods of time. The organism is apparently unable to utilize the minute quantities of iodine absorbed through the integument without the aid of the thyroid mechanism to collect and store it, thus preventing its passage through the body unused. This is good evidence that larvæ with intact thyroid apparatus are capable of utilizing very much smaller quantities of elemental iodine than are thyroidless animals. The lower limit of the iodine concentration requisite for metamorphosis of normal larvæ was not determined, as 0.0000007666 and 0.0000003833 were the weakest used and both proved to be above the lower limit just necessary to induce metamorphosis. On the other hand, these same concentrations were too low to induce metamorphosis in thyroidless animals.

The determination of the exact amount of iodine absorbed by the

larvæ of any single culture during the course of the experiment was not attempted. Anuran larvæ apparently do not swallow much water, but are constantly absorbing it through the skin and eliminating it through the kidneys, thus a constant stream of water flows through the organism. It is surprising how much water a 6 mm. tadpole will absorb in 24 hours. Recently the writer⁵ extirpated the kidneys of tadpoles, eliminating the excretory function. The result was a generalized body edema of enormous proportions due to water absorption. It is very unlikely that inorganic iodine will be absorbed by the larvæ at the same rate as water.

Iodine has a much wider distribution in nature than is commonly supposed, and is not confined solely to marine waters and organisms, and the thyroid glands of vertebrates. This halogen in some form or other, and in small quantities, is present in most surface water, and practically all organic matter, provided a fair sized sample is analyzed. It is surprising how many common foodstuffs contain traces of iodine. The amounts are of course very small, and this fact has been the chief hindrance in the way of recognizing iodine as of common occurrence, for it is only within recent years that a method sufficiently delicate to detect very minute traces of this substance has been devised. With further refinement of technique it is safe to assume that iodine will be found to have a far wider distribution in nature, and to play a greater functional part in organisms than is at present conceded.

In marine organisms, both animal and plant, iodine is usually found in some form, and certain animals like coral are said to have a specific iodine metabolism. Among land vertebrates, iodine is not only found in the thyroid, but normally occurs in the blood and tissues. For instance, Kendall⁶ finds that the average iodine content of calves' blood is approximately 0.015 mg. per 100 cc. of blood. The amount in the tissues is somewhat greater, and in the liver is still greater than in the tissues. Other investigators have at various times reported the presence of iodine in the ovaries, adrenals, spleen, and salivary glands. The thyroids of infants a few weeks after birth are said to contain iodine, hence it is not improbable that human milk contains traces of this substance.

⁵ Swingle, W. W., *J. Gen. Physiol.*, 1918-19, i, 509. McClure, C. F. W., *ibid.*, 261.

⁶ Kendall, E. C., *Am. J. Physiol.*, 1919, xlix, 136.

There can be little doubt that the iodine of the thyroid is obtained by the organism from the food and water. Marine and Rogoff⁷ have clearly shown that the affinity of thyroid tissue for iodine is so great that practically all iodine injected into the organism by experimental methods is "fixed" in the thyroid within a few minutes. Their experiments explain why it is that considerably more iodine is required to induce metamorphosis in thyroidless frog larvæ than in animals possessing glands.

The importance of iodine in the economy of the organism is at present little understood and much underestimated. The writer holds the view that this halogen will eventually take rank along with chlorine, phosphorous, and other elements essential to the maintenance of normal metabolism, growth, and development. The almost universal occurrence of a thyroid mechanism among the vertebrates for the assimilation and utilization of iodine in minute quantities, points to the conclusion that there is a definite iodine metabolism in these forms, and that it is necessary for the normal functioning of vital processes.

That elemental iodine functions within the thyroidless organism as such, is highly improbable; it is far more likely that it is combined with other substances in the blood and tissues, though what these substances are is unknown, just as it is unknown how the presence of the thyroid affects the iodine. In either case, however, the result is the same in frog larvæ; *i.e.*, normal metamorphosis. The fact that more iodine is required to bring about metamorphosis in thyroidless animals than in normal ones may indicate either that the thyroid gland tissue has added something to the iodine, thereby increasing its physiological activity, or that the gland has functioned as a storage organ and fixed the iodine in greater quantities, thus preventing its passage through the organism unused. It may well be that both these possibilities are realized in normal animals with intact glands. It is to this latter view that the writer adheres.

A recent writer⁸ has stated that iodine has nothing to do with limb development in the larvæ of Urodela, and doubt is expressed that con-

⁷ Marine, D., and Rogoff, J. M., *J. Pharm. and Exp. Therap.*, 1916, viii, 439.

⁸ Uhlenhuth, E., *J. Gen. Physiol.*, 1918-19, i, 534.

ditions are different in anuran tadpoles. This writer says in this regard:

"It is remarkable, however, that in two so closely related groups of animals as the caudate and tailless batrachians, the same process should be caused by so different a mechanism. It is difficult to refute the suspicion that in the *Salientia* the development of the limbs may be initiated only indirectly by the iodine action, perhaps on account of some incidental anatomical structure which first must be broken down by the autolytic action of the iodine."

This statement should be of interest to students of anuran metamorphosis who have experimented with iodine, as it is a denial of the process, namely limb development, most obviously brought out by iodine administration in these forms. There can be no question that iodine has little or no effect upon limb development of urodeles, but also there can be no doubt that this same substance stimulates to a remarkable degree limb growth and formation in the Anura. Despite the close phylogenetic relationship between Urodela and Anura there exist some sharp distinctions between the larvæ of the two groups in regard to physiological responses to environmental conditions, and in developmental history. Metamorphosis in the Apoda and Urodela is restricted chiefly to the reduction of the gills, the closing of the clefts, and the loss of the gill chamber and the finny margin of the tail; but in frogs and toads, the change from the tadpole to the final form is a fundamental reorganization of the organism, involving practically every organ. The following quotation is from Boulenger:³

"In the newts the transformation is much more gradual (*i.e.*, than the metamorphic change in Anurans) without the intercalation of a tadpole stage. The final transformation in the Urodeles consists essentially in the loss of the gills and the development of the eye-lids. I may mention that although the larvæ of Urodeles are often called tadpoles, the term as applied to them is quite misleading, the larvæ of frogs having an altogether different form and organization."

Limb development in amphibians varies considerably in the different types. Thus, in most Anura, the limbs develop slowly and require months and years (*Rana catesbiana*) for complete development. In other forms, such for instance as the urodele, *Salamandra maculosa*, the young are born with legs.

Uhlenhuth regards gill reduction and shedding of the skin in urodeles as constituting metamorphosis. It is evident that such a con-

ception is not sufficiently broad to account for the fundamental changes incident to anuran transformation. For in these animals metamorphosis usually implies complete shedding of the tadpole characters, such as the fish-like tail, larval mouth, gills, intestinal hystolysis, and reorganization and development of the frog character by growth of limbs and other fundamental changes. Whether or not the autolytic action of iodine is responsible for all these body changes can only be determined by future investigation.

There are unquestionably other unknown chemical factors involved in the degenerative and regenerative processes involved in metamorphosis, and as investigation in this field has scarcely begun, all generalizations should be regarded as premature.

In conclusion the writer wishes to acknowledge his indebtedness to Professor E. N. Harvey and Professor E. G. Conklin for valuable criticism and advice.

INFLUENCE OF THE CONCENTRATION OF ELECTROLYTES ON THE ELECTRIFICATION AND THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES.

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(Received for publication, September 6, 1919.)

I. Influence of the Concentration of Electrolytes upon the Rate of Diffusion of Positively Charged Particles of Water through Collodion Membranes.

When we fill a collodion flask, as described in a preceding publication,¹ with a solution of a non-electrolyte, *e.g.* cane sugar, grape sugar, or glycerol, and dip the flask into a beaker containing distilled water, the level of the liquid in the flask will rise, as is to be expected on the basis of the gas pressure theory of osmosis. When we close the opening of the collodion flask with a rubber stopper, perforated by a glass tube with a bore of about 2 mm. in diameter serving as manometer, the rate of diffusion of water into the solution can be conveniently followed. Since at the same time sugar will diffuse out of the flask into the surrounding distilled water, the rise of the column of liquid in the manometer will cease after some time (*e.g.* after about 70 minutes at 24°C.) and will be followed by a fall in the level of the liquid, until finally the solutions inside and outside the collodion flask become identical. It is therefore necessary to consider only the initial rise of liquid in the manometer as an indicator for the attractive action of the solution upon water, and in all the figures given in this paper the readings were made 20 minutes after commencement of the experiment. At the beginning of the experiment the level in the manometer was usually about 30 mm. above that of the distilled water in the beaker in order to discover possible cases of negative osmosis. All collodion flasks had practically the same sur-

¹ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 717.

face, being cast inside of the same Erlenmeyer flask, and during the experiment the whole area of the flask was submersed in the beaker. The temperature was always 24°C.

In Fig. 1 the ordinates are the values for the rise in the level of the solution in the glass tube (after the first 20 minutes) which occurred when the collodion flasks filled with different concentrations of cane sugar, grape sugar, or glycerol were dipped into beakers containing distilled water. The abscissæ are the logarithms of the concentration of the sugar solution. The reader will notice that for concentrations below $M/64$ the curves run practically parallel to the base line while a sharp rise begins at about $M/16$ or $M/8$. If the rise is plotted over the concentration (instead of over the logarithms of the concentration) the curve is almost a straight line between concentrations of $M/32$ and $1 M$ (Fig. 2), as was to be expected on the basis of the gas pressure theory. When, however, we make the same experiments with solutions of *electrolytes*, separating them from pure water by collodion membranes, a curious phenomenon is observed, which was partly described in the first paper; namely, that at a very low concentration of electrolyte the rate of diffusion of water through the collodion membrane from pure solvent into the solution increases rapidly with increasing concentration and that it reaches a maximum at a comparatively low concentration¹ of the electrolyte. It is easier to follow the facts to be described with the help of the curves given in Fig. 3. The abscissæ are the logarithms of the concentration of the solution, while the ordinates give the height to which the liquid in the manometer has risen in 20 minutes. The reader will notice that the curves for five sodium salts are given— $NaCl$, Na_2SO_4 , Na_2 oxalate, Na_3 citrate, $Na_4Fe(CN)_6$. The solutions must not be acid for this experiment, and the hydrogen ion concentration of the solutions of $NaCl$, Na_2SO_4 , and Na_2 oxalate was almost that of the point of neutrality (pH about 6.0, or between 6.0 and 7.0), while the two other solutions were slightly alkaline. Beginning with the lower concentrations of the solutions of these salts the curves rise sharply with the increase in concentration, reach a maximum at a concentration of about $M/256$, and then with a further increase in concentration the curves fall abruptly to reach a minimum, varying for the different salts between $M/32$ and $M/8$. After this the curves rise again.

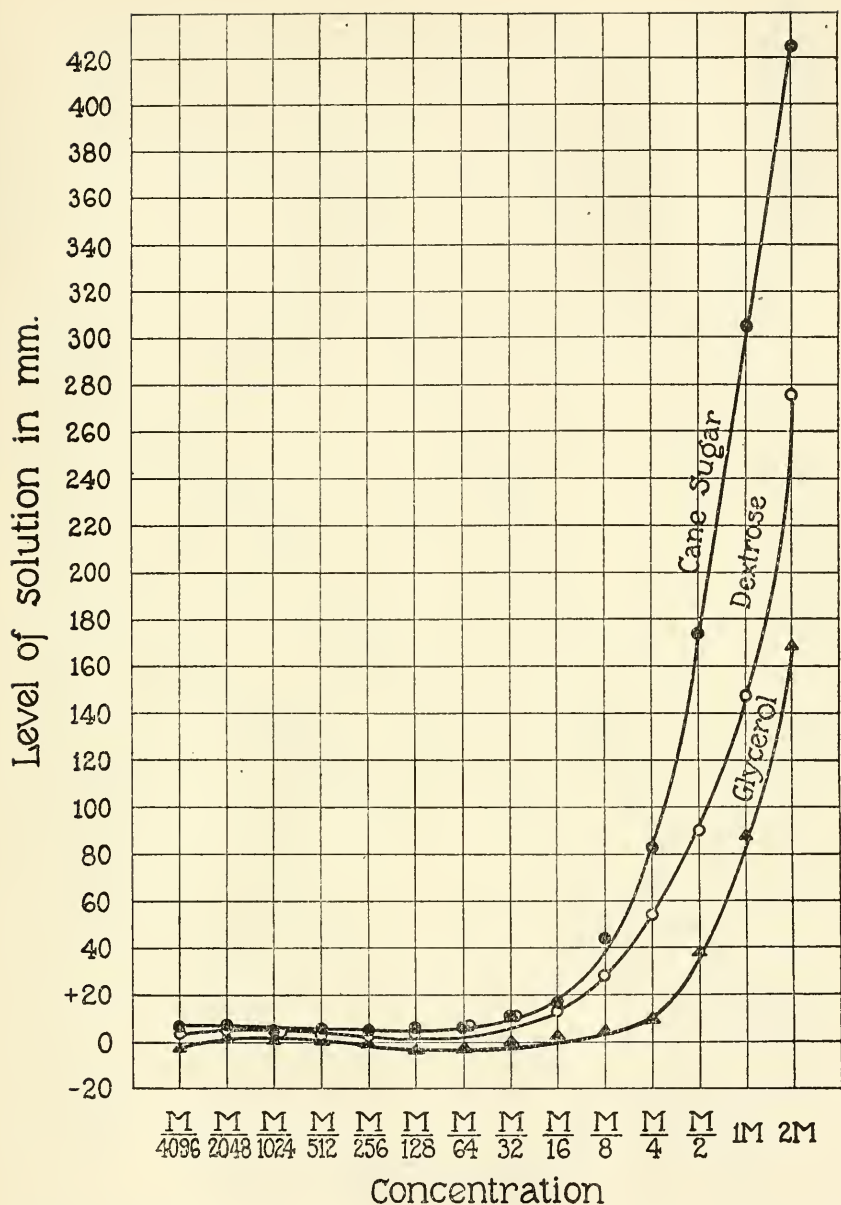


FIG. 1. Influence of concentration of non-electrolytes on initial rate of diffusion of water through a collodion membrane from pure solvent to solution. Abscissæ are logarithms of concentrations, ordinates the rise in mm. in level of solution in the manometer tube after 20 minutes.

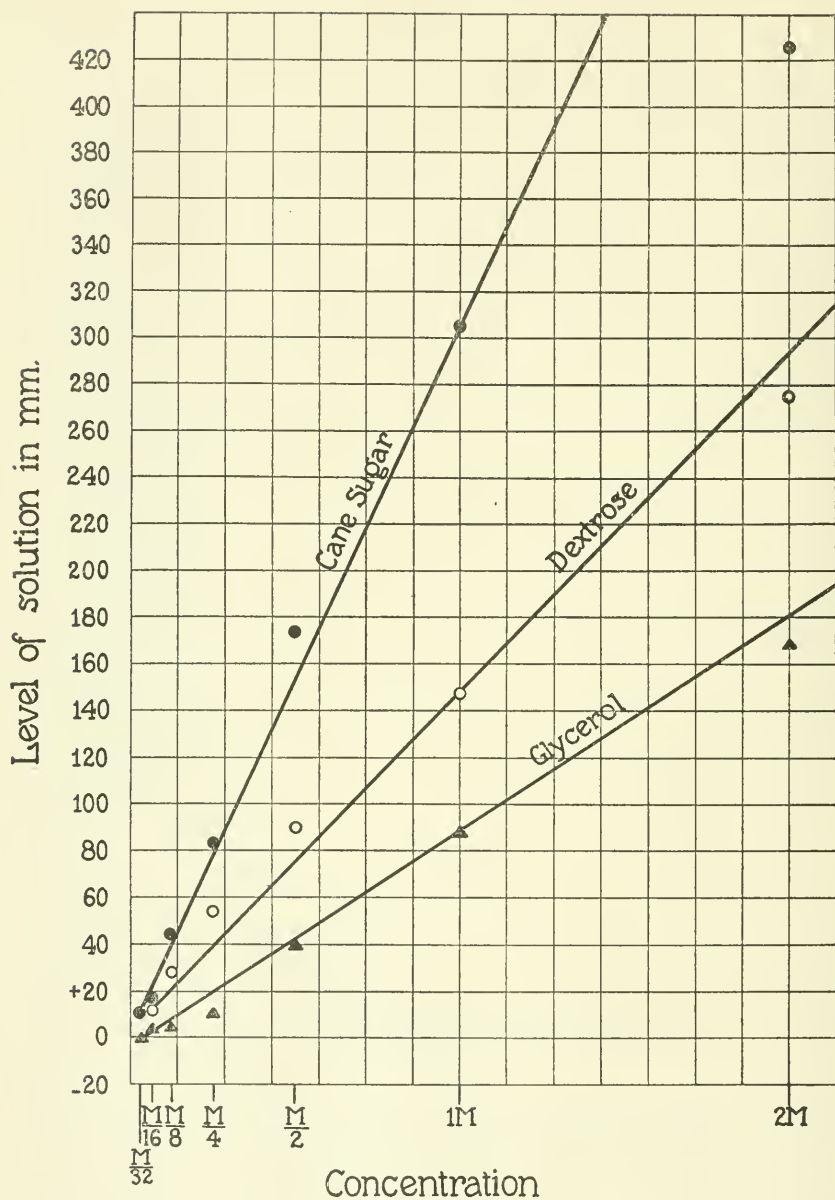


FIG. 2. The same as in Fig. 1, except that abscissæ in Fig. 2 are the concentrations instead of the logarithms of concentrations as in Fig. 1. Within concentrations of $\frac{M}{32}$ and $1M$ the initial rise of liquid in 20 minutes is in direct proportion to the concentration, as van't Hoff's law demands.

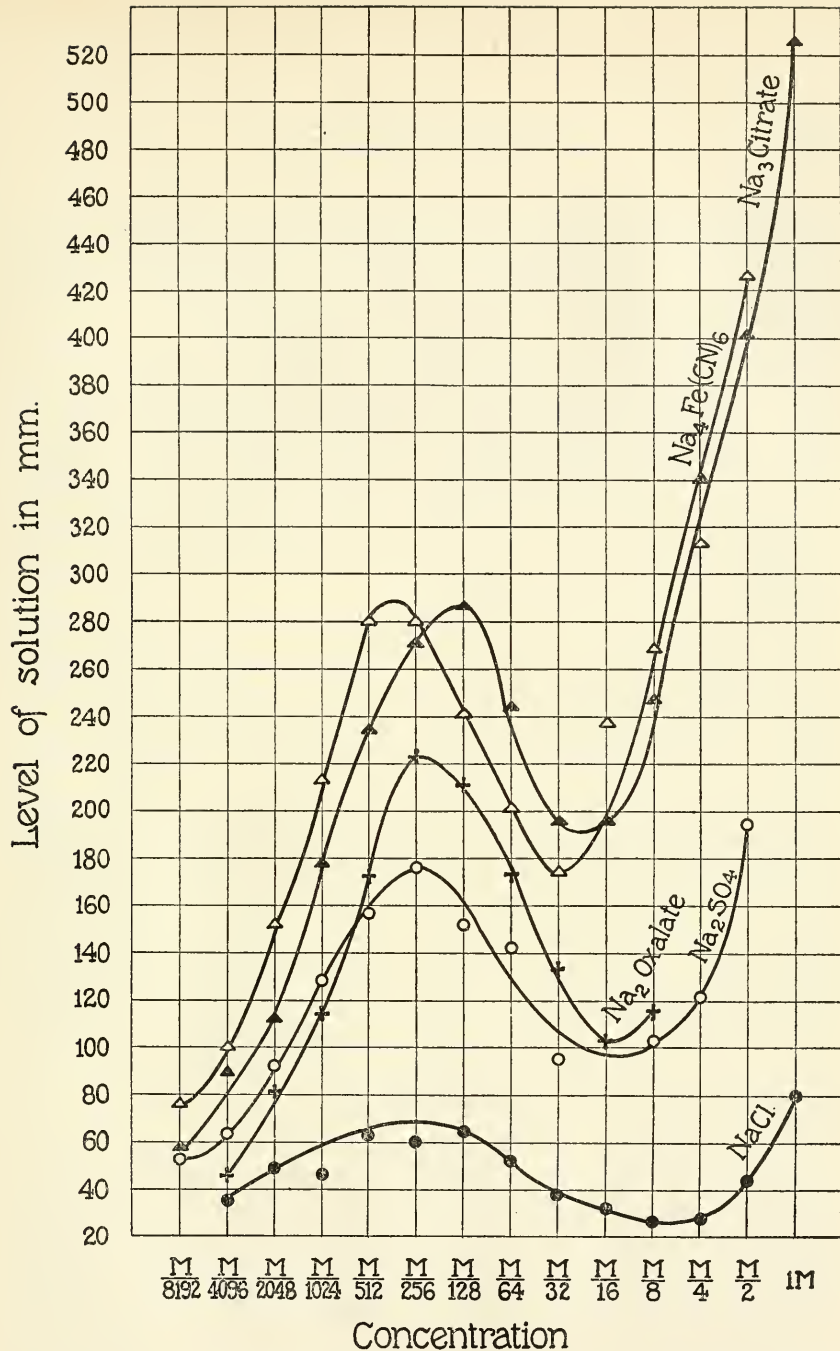


FIG. 3. Curves representing influence of concentration of five different sodium salts upon initial rate of diffusion of water through a collodion membrane from pure solvent to solution. Abscissæ are the logarithms of concentration. Ordinates represent level of solution in manometer tube after 20 minutes. The curves rise steeply until the concentration of $M/256$ is reached, then, with further increase in concentration, the curves drop, and rise again when the concentration is between $M/16$ and $M/4$.

While the ordinates of these curves represent the height in the manometer 20 minutes after the beginning of the experiments, the same form of curves is obtained when the readings are taken after 5, 10, or 30 minutes.

The interpretation of this complicated system of curves becomes simplified if we compare it with the curves for non-electrolytes in Fig. 1. We then notice that the character of both sets of curves agrees in the region of the higher concentrations above from $M/32$ to $M/4$, and we are therefore inclined to assume that the second rise in the curves for solutions of sodium salts beginning with $M/32$ to $M/4$ (according to the nature of the salt in solution) is due mainly to the gas pressure effect of the solution.

The abrupt rise and fall of the curves in Fig. 3 for lower concentrations than $M/32$ are not repeated in the case of the curves for the non-electrolytes in Fig. 1, and we are inclined to ascribe these peculiarities of the curves to the electrical action of the ions on the rate of diffusion of the electrified particles of water. Fig. 4 expresses this division of the curves diagrammatically.

In a preceding paper¹ we have shown that in the presence of neutral (or alkaline) salts with monovalent or bivalent cation the particles of water diffuse through the membrane as if they were positively charged, being attracted by the anion of the salt and repelled by the cation with a force which increases with the number of charges of the ion (and inversely with a quantity which we arbitrarily designated as the "radius" of the ion). The source of the electrification of the water particles as well as the specific mechanism by which the ions of the solution influence the rate of diffusion of water will not be discussed in this paper. It will, however, simplify the presentation of our experiments if it be permitted to describe our results as if the charged water particles were attracted or repelled electrostatically by the ions of the solution. We notice that in Fig. 3 the curves rise the more rapidly with increasing concentration the higher the valency of the anion of the salt, and with the reservation just mentioned we may ascribe this initial steep rise in the curves between a concentration of the solution of 0 to a concentration of $M/256$ to the fact that the attractive action of the anion of the sodium salts upon the rate of diffusion of the positively charged particles of water through the mem-

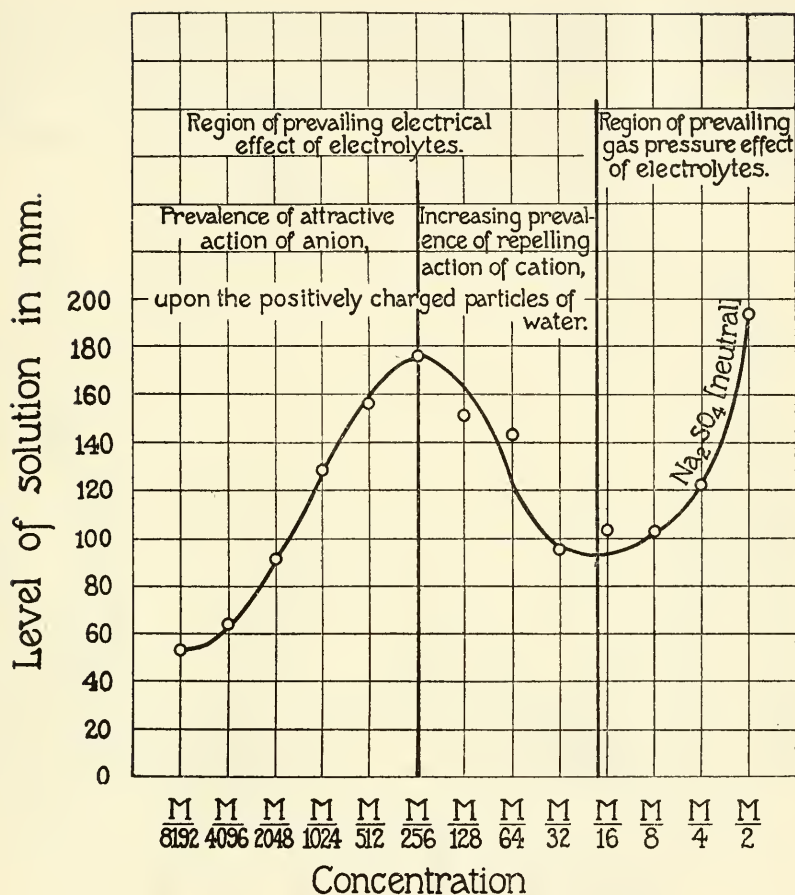


FIG. 4. Explanation of the curves in Fig. 3 given for the Na₂SO₄ curve. The first rise and fall between M/8,192 and M/16 represent the electrostatic action of ions of the solute upon the rate of diffusion of positively charged water particles. The second rise beyond M/16 represents the rise due to the gas pressure effect of the solute. The steep rise of the curve between 0 and M/256 is due to the prevalence of the attractive action of anion upon the positively charged molecules of water, while the drop beyond M/256 is due to the fact that the repelling action of cation upon the positively charged particles of water increases more rapidly with increasing concentration than the attractive action of SO₄ for the water.

brane is greater than the repelling action of the cation. The curves reach a maximum when the solution reaches a molecular concentration of about $M/256$ and from now on the curves drop rapidly with a further increase in the concentration of the solution. We assume that when the concentration of the solution of the sodium salts reaches a certain value the repelling action of the cation—in this case Na—

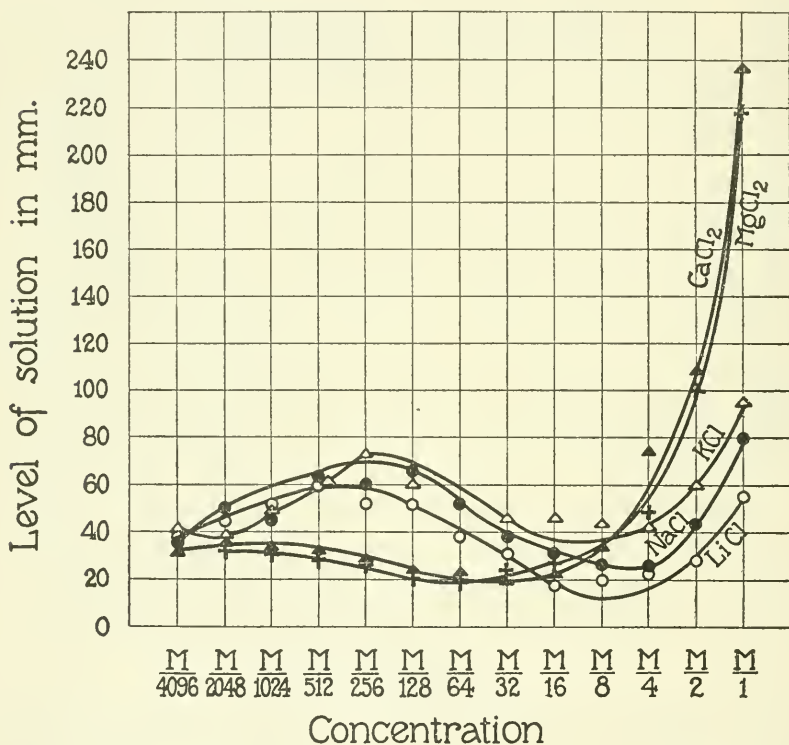


FIG. 5.

increases more rapidly with increasing concentration than the attractive action of the anion upon the positively charged particles of water. This drop, however, is comparatively smaller the higher the valency of the anion. In the case of sodium chloride (Fig. 3) the curve is lower at $M/8$ (the minimum) than at $M/4,096$, while for SO_4 and oxalate the minimum reached by the curves (at $M/16$) is considerably above that

reached by the NaCl curve, and the minimum for the citrate and ferrocyanide is still higher. This was to be expected from the fact that the attractive action of the anion upon the positively charged particles of water is higher the greater the number of charges or the valency of the anion.

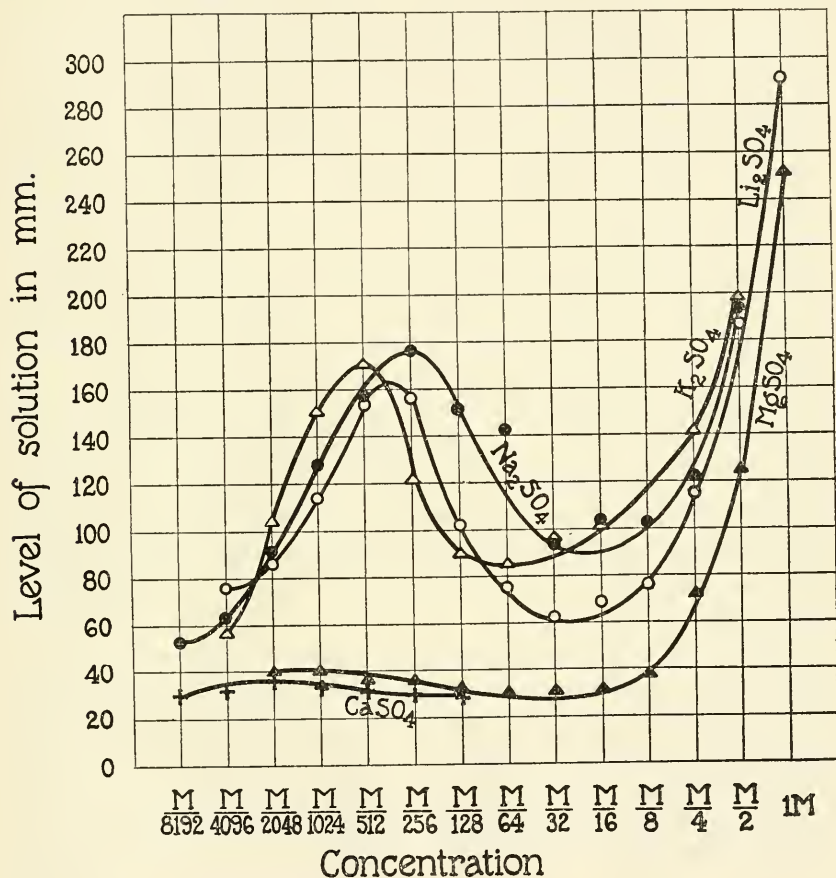


FIG. 6.

FIGS. 5 and 6. Curves representing influence of different chlorides (Fig. 5) and sulfates (Fig. 6) on initial rate of diffusion of water from pure solvent into solution, showing that the drop is greater in case of Li than of Na, or of K, and supporting the view that the drop is due to the repelling effect of the cation upon the positively charged particles of water.

The facts given are not peculiar to sodium salts but can be demonstrated in the case of all neutral or alkaline solutions of salts with monovalent or bivalent cation. Fig. 5 gives the curves for different chlorides and Fig. 6 for different sulfates. All the curves for these salts rise up to a concentration of about $M/256$ and then fall and finally rise again when the concentration is so high that the gas pressure effect begins to obliterate the electrical effect of the ions. The turning point for the commencement of the prevalence of the gas pressure effect is at a lower gram-molecular concentration for the sulfates than for the chlorides; Fig. 3 shows that the concentration of the turning point is the lower the higher the valency of the anion. It may be that this is simply the consequence of the fact that the higher the valency of the anion the greater the number of ions into which the salt dissociates electrolytically, but it is not yet certain that this is the full explanation. Another fact, however, is certain; namely, that the depression of the curves when the concentration exceeds $M/256$ is greater for the lithium salts than for the sodium salts, and greater for the sodium salts than for the potassium salts. This was to be expected if the drop is due to the repelling action of the cation on the positively charged particles of water, since the repelling action of cations on the positively charged particles of water increases inversely with a quantity which we arbitrarily designate as the "ionic radius." This "ionic radius" is less for Li than for Na and presumably less for Na than for K.

In the case of neutral solutions of salts with bivalent cation the repelling action of the cation is so great that it balances or exceeds the initial attractive action of a bivalent or monovalent anion for positively charged particles of water; the depressing action of the cation in concentrations above $M/256$ upon positively charged particles of water remains, however, noticeable especially in the SO_4 curves.

II. Influence of the Concentration of Electrolytes upon the Rate of Diffusion of Negatively Charged Particles of Water through Collodion Membranes.

The curves given in Figs. 3 to 6 contain a paradoxical fact, which can be expressed as follows: *when solutions of neutral or alkaline salts with monovalent or bivalent cation are separated by a collodion membrane*

from pure water the attraction of the solution for water diminishes with increasing concentration for concentrations between $M/256$ to about $M/32$ or above, according to the nature of the electrolyte. This is exactly the reverse of what we should expect on the basis of van't Hoff's law which demands that the attraction of the solution for water should increase with the concentration. Such a reversal occurs only in the case of electrolytes and we assume provisionally that it is due to the fact that the repelling action of the cation of the electrolyte upon the positively charged particles of water increases inside the critical range of concentrations more rapidly with the concentration than the attractive action of the anion upon the same particles of water.

It will be necessary to test this assumption by further experiments.

We have shown in the first paper that water particles diffuse through collodion membranes in the form of *positively* charged particles from pure solvent to solutions of electrolytes with monovalent or bivalent cation when the solution is neutral or alkaline in reaction. When, however, the solution is rendered sufficiently acid the water particles diffuse through the membrane as if they were *negatively* charged, being attracted by the cation and repelled by the anion of the electrolyte with a force increasing with the valency of the ions. We will now consider the influence of the concentration of electrolytes upon the rate of diffusion of *negatively* charged particles of water through the membrane.

We have stated in a former paper² that the collodion membranes were suspended for 1 night in a 1 per cent gelatin solution before being used; after this they were used sometimes for a week or more without any further gelatin treatment. The membranes were, of course, rinsed a large number of times in warm water after the gelatin treatment to remove all the gelatin that could be removed by rinsing. This gelatin treatment is not necessary to obtain the results with neutral solutions of electrolytes described in the preceding chapter. The gelatin treatment of the membrane is, however, necessary to obtain the results with negatively charged particles of water to be described in this chapter. We intend to return to this fact in a later paper.

² Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 87.

Negatively charged particles of water are attracted powerfully by bivalent or polyvalent cations and repelled powerfully by bivalent or polyvalent anions. We may, therefore, expect that when we make a CaCl_2 solution sufficiently acid through the addition of HNO_3 or HCl the curves showing the influence of the concentration of the electrolyte on the rate of diffusion of water should be similar to the curves in Figs. 3, 4, and 6, representing the influence of different concentrations of neutral solutions of Na_2SO_4 upon the rate of diffusion of positively charged particles of water through the same membranes. Fig. 7 shows that this is correct. The two upper curves represent the influence of variations in the concentration of CaCl_2 or $\text{Ca}(\text{NO}_3)_2$ dissolved in $\text{M}/1,024$ or $\text{M}/128$ HNO_3 or HCl . These acid solutions of CaCl_2 or $\text{Ca}(\text{NO}_3)_2$ cause a negative electrification of the water particles diffusing through the membrane. The attraction of the negatively charged water particles by Ca ions increases with an increase in the concentration of the calcium salt up to $\text{M}/64$, when the curve falls until the concentration of the calcium salt is $\text{M}/4$ where a minimum is reached. After this the gas pressure effect makes itself felt and the curve rises rapidly with rising concentration. In this case the drop in the two curves for the acid solutions when the concentration of the calcium salt exceeds $\text{M}/64$ is due to the fact that beyond a certain concentration of the calcium salt the repelling effect of the anion (Cl and NO_3) upon the negatively charged particles of water increases more rapidly with increasing concentration than the attractive action of the Ca ion.

The fact that the curve for $\text{Ca}(\text{NO}_3)_2$ in $\text{M}/128$ HCl is slightly lower than the curve for CaCl_2 in $\text{M}/1,024$ HNO_3 is due to the "negative osmosis" caused by acid solutions of sufficient concentration, as will be shown in the next chapter.

The three lower curves of Fig. 7 represent the influence of various concentrations of CaCl_2 upon positively charged particles of water and these curves are similar to those given for the effect of neutral solutions of CaCl_2 in Figs. 5 and 6. When CaCl_2 is dissolved in $\text{M}/128$ KOH the rise and drop of the curve become a little more marked than when the solution of CaCl_2 is neutral. The reason for this will become clear through the discussion in the next chapter of the phenomenon of negative osmosis caused by $\text{Ca}(\text{OH})_2$.

When we render Na_2SO_4 solutions acid (by dissolving the salt in $\text{M}/128$ or $\text{M}/1,024$ HNO_3) the negatively charged particles of water diffusing through the membrane will be repelled by the SO_4 ion and attracted by both the Na and H ions. Since the SO_4 ion carries two

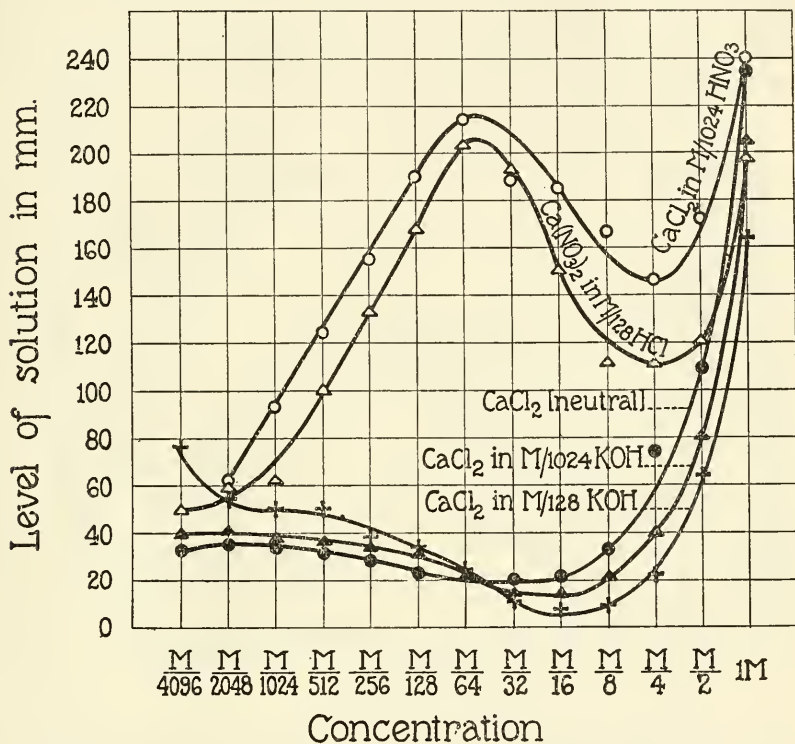


FIG. 7. Upper two curves representing influence of acid solutions of CaCl_2 or of $\text{Ca}(\text{NO}_3)_2$ upon negatively charged particles of water. At first there is a steep rise of the curve due to prevalence of attraction of Ca for the negatively charged particles of water, then beyond $\text{M}/64$ a drop in the curve due to prevalence of repelling action of NO_3 and Cl upon the negatively charged particles of water. No initial rise in the three lower curves, since in neutral and alkaline solutions of Ca water diffuses in the form of positively charged particles which are too strongly repelled by Ca to permit an initial rise in the level of the solution.

charges while the cations Na and H are monovalent the greater electrostatic action of the SO_4 ion should inhibit the electrostatic attraction of water by such solutions in the same way as is done by neutral so-

lutions of CaCl_2 in the case of positively charged particles of water. Fig. 8 shows that this is the case. The two lower curves in Fig. 8 show the influence of concentrations of sufficiently acid solutions of Na_2SO_4 upon the rate of diffusion of water from distilled water into

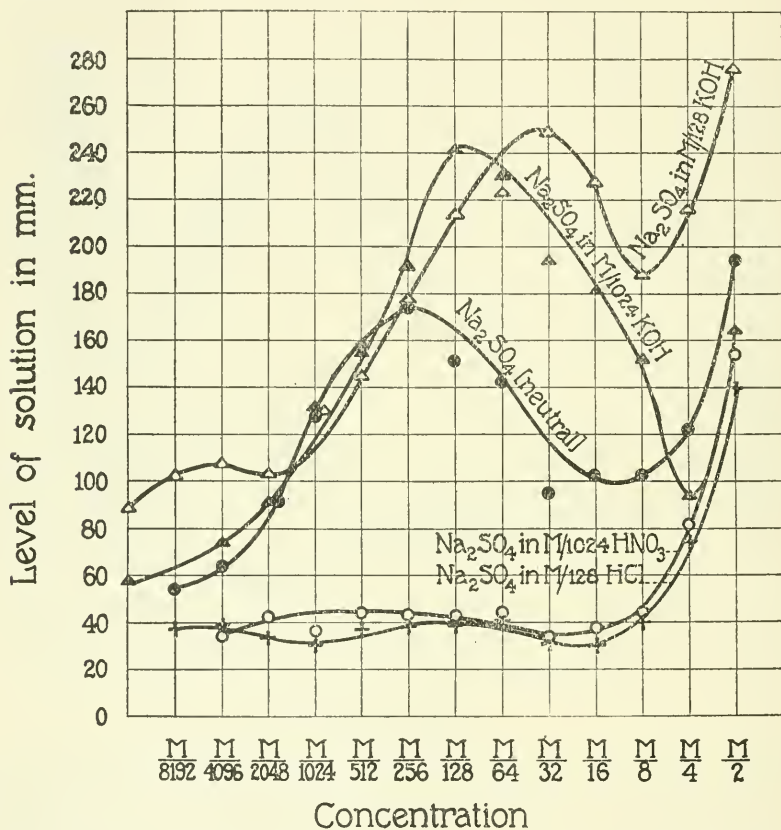


FIG. 8. The curves for solutions of Na_2SO_4 when sufficiently acid show no initial rise, since the negatively charged particles of water are too strongly repelled by the SO_4 ions.

solution. The curves resemble the curves for the effect of different concentrations of non-electrolytes, except for an intimation of a slight drop for concentrations of Na_2SO_4 between $\frac{M}{64}$ and $\frac{M}{8}$. The three upper curves represent the influence of concentrations of different

neutral and alkaline solutions of Na_2SO_4 upon the rate of diffusion of positively charged water particles.

It was finally expected that the curves representing the influence of various concentrations of NaCl upon the rate of diffusion of water through collodion membranes should be similar in neutral, acid, and alkali solutions, for the reason that anion and cation of the salt are

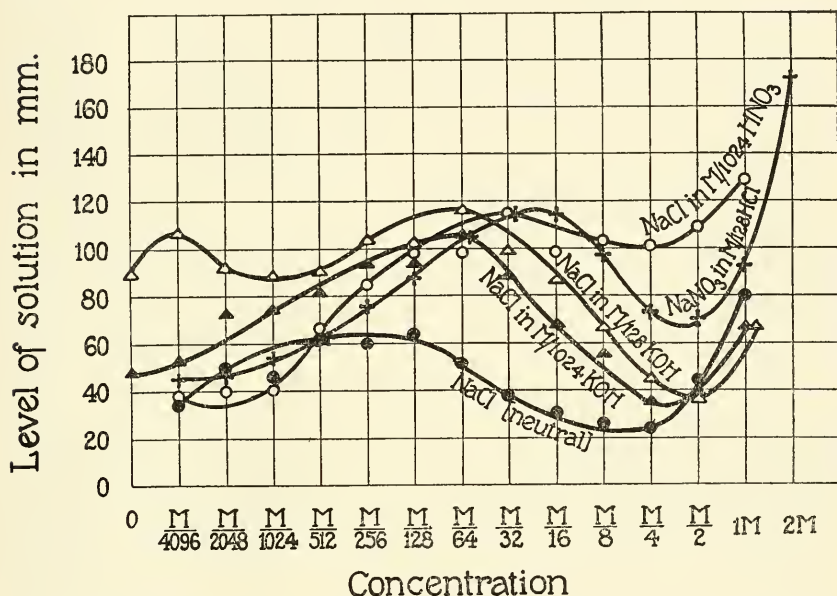


FIG. 9. Salts with monovalent anion and cation, like NaCl , show both rise and drop in alkaline, neutral, and acid solutions. In neutral and alkaline solutions the first rise is due to the attractive action of the Cl ion and the drop to the repelling action of the Na ion upon the positively charged particles of water. In acid solution the first rise in the curve is due to the attractive action of the Na ion and the drop to the repelling action of the Cl ion upon the negatively charged particles of water.

both monovalent (Fig. 9). In the presence of a sufficiently acid solution of NaCl the particles of water diffuse as negatively charged molecules through the membrane which are attracted by the Na ions and repelled by the Cl ions. The initial rise of the curve is due to the attractive action of the Na ion, and the drop in the curve is due to the increasing prevalence of the repelling action of the Cl ion. In neutral

and alkaline solutions of NaCl the Cl ion attracts the water (which is charged positively) and the fall of the curve is due to the Na ion. The complete suppression of the rise which is caused in CaCl_2 when the solution is neutral or alkaline, and in Na_2SO_4 when the solution is acid, is due to the fact that in these cases the attracting ion is mono-

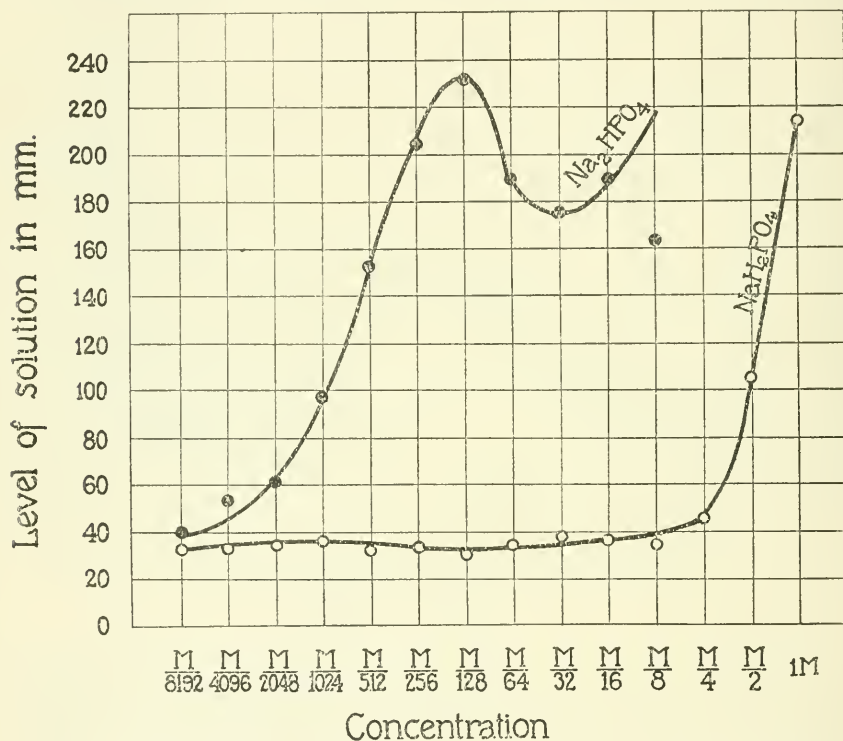


FIG. 10. The Na_2HPO_4 curve shows the initial rise and drop since the water diffuses in the presence of this salt in the form of positively charged particles which are attracted by the trivalent PO_4 ion and repelled by the monovalent Na and H ions. The NaH_2PO_4 curve shows no initial rise, since in the presence of this salt water diffuses in the form of negatively charged particles which are repelled by the trivalent PO_4 ion and attracted by the monovalent Na and H ion.

valent and the repelling ion bivalent. Fig. 9 shows that the curves representing the influence of the concentration of NaCl solutions upon the rate of diffusion of water are similar for neutral, alkaline, and acid solutions of NaCl. The drop is more considerable for NaNO_3 solu-

tions in $M/128$ HCl than in $M/1,024$ acid, on account of the negative osmosis caused by solutions of acids, as the next chapter will show.

Fig. 10 shows the difference in the influence of concentration upon the rate of diffusion of water in the case of Na_2HPO_4 and NaH_2PO_4 . In the presence of the latter solution the water diffusing through the membrane is negatively charged and the curve does not show the initial rise and drop but shows only the gas pressure effect of the solution. In the case of Na_2HPO_4 water diffuses in the form of positively charged particles and the curve shows the rise and drop as described for neutral and alkaline salts in which the anion has a higher valency than the cation.

We can therefore state that for the diffusion of negatively as well as positively electrified particles of water the law of van't Hoff is reversed within a certain range of concentrations of electrolyte—in the extreme between about $M/256$ and $M/4$ —inasmuch as in this range the attraction of a solution of an electrolyte for pure water diminishes with increasing concentration.

III. Negative Osmosis.

Dutrochet,³ Graham,⁴ and Flusin⁵ have observed a striking phenomenon of negative osmosis in certain acids; namely, that the stream of liquid diffuses through pig's bladder from acid to pure water, instead of in the opposite direction. This was observed for tartaric and oxalic acids. Flusin tries to explain the phenomenon on the basis of a difference in degree of imbibition or swelling of the two sides of pig's bladder, one side being in contact with pure water where the swelling is slight, the other being in contact with acid where the swelling is great. Bartell, and Bartell and Hocker⁶ have observed negative osmosis through porcelain even in the case of some neutral

³ Dutrochet, H., *Ann. chim. et. phys.* 1835, lx, 337.

⁴ Graham, T., *Phil. Tr.*, 1854, cxliv, 177.

⁵ Flusin, G., *Ann. chim. et phys.*, 1908, xiii, 480. For a discussion of the literature see Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipsic, 4th edition, 1914, 248.

⁶ Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036.

salts. The writer has never observed negative osmosis when solutions of neutral salts were separated from pure solvent by collodion membranes.

The writer has recently investigated the influence of the concentration in acids and alkalies on the rate of diffusion of water through collodion membranes (previously treated with gelatin), with the result that the phenomenon of negative osmosis described for tartaric and oxalic acids is very common in acids and alkalies, that it *occurs in exactly the same range of concentration where the drop in the curves of the neutral salt solutions occurs, namely within a range between $M/256$ to about $M/4$, and that the phenomenon is in reality nothing else but this drop*. The difference between the nature of the drop in the case of solutions of neutral salts on the one hand and of solutions of acids and alkalies on the other is that in the case of alkalies and acids the drop is not only relative but absolute. Titration experiments show that acid diffuses from the acid solution into distilled water and that the concentration of acid in the solution is considerably less after 20 minutes than at the beginning. Since at the same time the total volume of solution diminishes—this being the nature of negative osmosis—we must conclude that the diminution of the volume of the solution is due to the fact that the combined volume of acid and water diffusing out from the solution is slightly larger than the volume of water diffusing simultaneously into the solution. We shall return to this problem in a subsequent paper. Since acids as well as bases diffuse into the pure solvent the phenomenon of negative osmosis can only be observed during a short period at the beginning of the experiment.

In all the experiments described in the preceding pages it was necessary to put the solution into the collodion flask and to dip the latter into distilled water in order to observe the influence of the solution on the initial rate of diffusion of water. More water diffuses into the solution than diffuses simultaneously in the opposite direction and as a consequence the level of the water in the glass tube rises. When we put solutions of certain acids or alkalies into the collodion flasks and dip these flasks into distilled water the level of the liquid in the manometer falls instead of rising. If, however, we put the solution of the same acids or alkalies outside, filling the collodion flask with

distilled water, we observe an initial rise of liquid in the manometer. For these experiments with acids, the collodion membranes must be bathed once over night in a 1 per cent gelatin solution. Fig. 11 shows the influence of the nature and concentration of acid upon the phenomenon. Since we are dealing here with a loss of volume on the

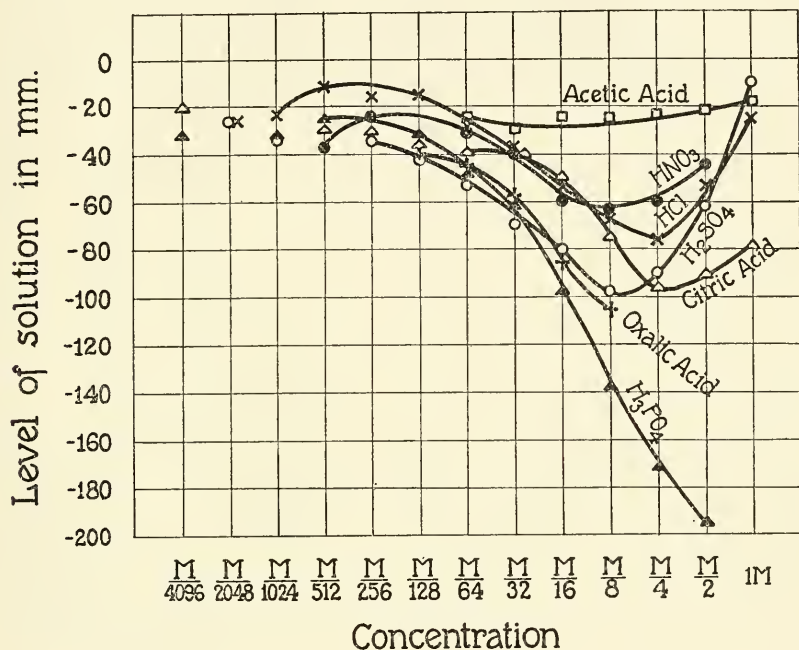


FIG. 11. Negative osmosis in acids, showing that the drop occurs here as in all other cases in a range of concentrations between $M/256$ and $M/8$ or $M/4$. The drop is greater for acids with bivalent or trivalent anion than for acids with monovalent anion, showing that the drop is due to the repelling action of the anion upon the negatively charged particles of water.

part of the solution, we must plot the rise observed in the manometer as a negative quantity below the zero line. The curves show that the drop is confined to the same range of concentrations where a similar drop occurs in the case of the neutral salts. The level of the water in the manometer tube was at the beginning of the experiment about 30 mm. When the flasks thus filled with distilled water were put

into beakers containing solutions of acid the level of the pure water in the flask rose during the first 20 minutes or more whenever the concentration of the acid was $M/256$ and the rise increased with an increase in the concentration until the concentration was about $M/4$. Then the level of the water inside the flask fell again.

In the presence of acids water diffuses through the membrane in the form of negatively charged particles. We have assumed that the drop is due to the repelling action of that ion of the electrolyte which has the same charge as the water particles; *i.e.*, the anion in the case of acids. Our assumption is supported by the fact that aside from the degree of electrolytic dissociation the drop increases with the increase of valency of the anion of the acid as we should expect. The drop is least in the case of the acids with monovalent anion (NO_3 and Cl), is greater in the case of SO_4 and oxalate anion, and still greater in the case of PO_4 . The drop in citric acid is a little less than in oxalic acid, as is to be expected from the fact that citric acid is a weak acid. In the case of acetic acid no rise and drop in the curve is noticeable except the rise due to the gas pressure effect of the solution.

In the case of alkalies we are restricted to low concentrations by the fact that they dissolve the collodion membranes when the concentration becomes moderately strong. As Fig. 12 shows, we observe absolute negative osmosis in the case of $\text{Ca}(\text{OH})_2$ and $\text{Ba}(\text{OH})_2$ when the concentration of the solution exceeds $M/256$. In the case of alkalies with monovalent cation the usual drop in the curves—*i.e.* relative negative osmosis—was observed, but not an absolute negative osmosis; *i.e.*, an absolute diminution of the volume of the solution.

The negative osmosis in the case of acids and alkalies becomes absolute for the reason that the initial rise in the curve due to the attractive action of the ion with the opposite sign of charge from that of the water is too slight or entirely lacking in the case of the acids or alkalies.

The writer was interested in finding out whether aluminium salts with bivalent or trivalent anion induce negative osmosis. In Fig. 13 are plotted the curves representing the influence of different concentrations of Al_2Cl_6 , $\text{Al}_2(\text{SO}_4)_3$, and aluminium citrate on the rate of diffusion of water through a collodion membrane. All the solutions had about the same hydrogen ion concentration and the water diffused

through the membrane in the form of negatively charged molecules, which according to our theory are attracted by the Al ion and repelled by the anion, the repelling action increasing with the valency of the anion. The curves show that Al_2Cl_6 attracts water very violently, $\text{Al}_2(\text{SO}_4)_3$ much less, and that aluminium citrate acts like a solution of a non-electrolyte, the attractive and repelling action of the two trivalent ions, Al and citrate, balancing each other. But no negative osmosis was noticed. This shows that the electrostatic effect of the H ion upon the rate of diffusion of water is considerably smaller than

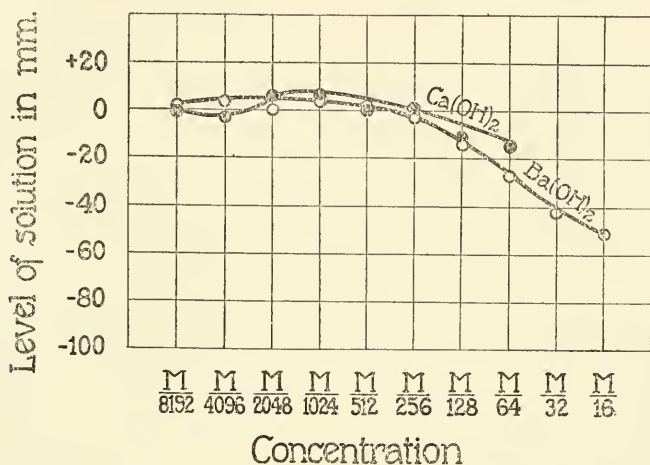


FIG. 12. Curves showing negative osmosis in the case of Ca(OH)_2 and Ba(OH)_2 occurring in that range of concentrations where the drop in Fig. 3 occurs.

the electrostatic action of trivalent cations like Al, while the electrifying effect of the H ion upon the water seems to be more nearly equal to the electrifying effect of the trivalent cation upon water. This suggests that the electrifying effect of ions upon water and their electrostatic effect upon the rate of diffusion of water are due to different qualities of the ion.

The curves for aluminium salts in Fig. 13 show no drop. As a matter of fact, a slight drop can be demonstrated in the AlCl_3 curves but in concentrations higher than those in Fig. 13, namely about M/32 or M/16, a phenomenon which finds its explanation perhaps in the fact that the cation is trivalent and the anion only monovalent.

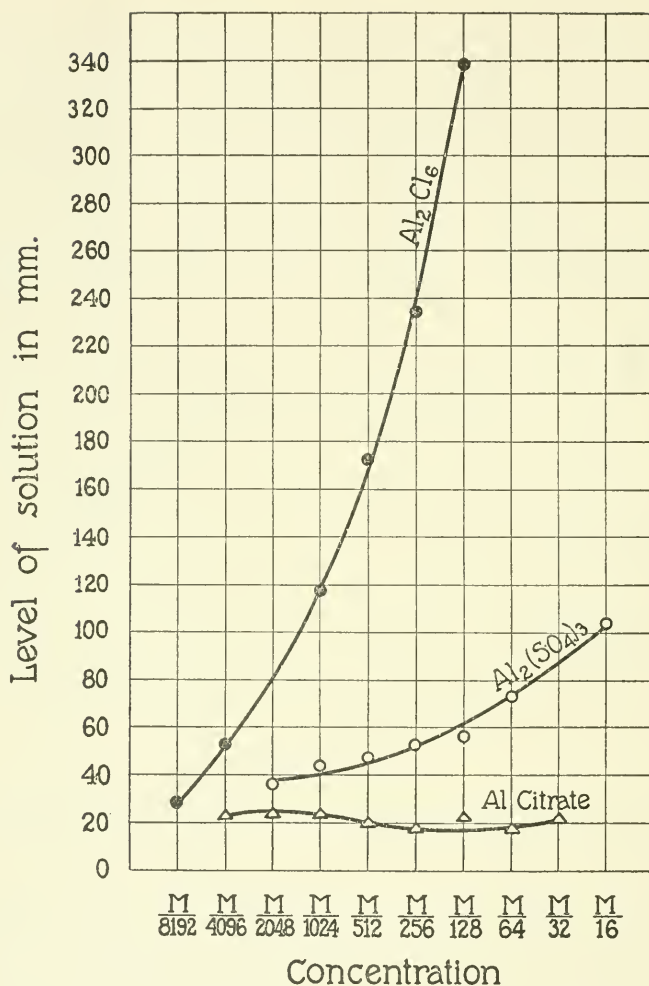


FIG. 13. Curves representing the attraction of different aluminium salts for negatively charged particles of water. The attraction is a maximum in the case of Al_2Cl_6 , is less in the case of $Al_2(SO_4)_3$, and is practically lacking in the case of aluminium citrate, showing the increase in the repelling effect of the anion upon the negatively charged particles of water with increasing valency of the anion.

IV. Further Proof that the Negative Osmosis Is Due to the Repelling Action of the Ion with the Same Sign of Charge as that of the Electrified Water Particles.

When we fill the collodion flask with a $M/256$ solution of Na_2SO_4 (made neutral or slightly alkaline) and dip the flask into distilled water, we notice a rather high initial rate of diffusion of water into the flask caused by the fact that the attraction of the SO_4 ion with its two charges upon the positively charged particles of water is greater than the sum of the repelling action of the two Na ions. In higher concentrations of Na_2SO_4 this difference becomes less (Fig. 4), as we assume through the fact that for some reason the repelling action of the Na ions on the positively charged water particles increases more rapidly with further increase in concentration than the attractive action of SO_4 . If this assumption is correct the addition of a salt of the type $MgCl_2$ to a $M/256$ solution of Na_2SO_4 should lower the rate of diffusion of water into the $M/256$ solution of Na_2SO_4 more than the addition of the same concentration of KCl ; since the repelling effect of the bivalent Mg ion upon the positively charged water particles is greater than the repelling effect of the monovalent K ions. The addition of $LiCl$ should lower the rate of diffusion of water more than the addition of KCl but less than $MgCl_2$, since the electrostatic effect of Li is greater than that of the K ion but less than that of the Mg ion. Fig. 14 shows that this is actually true. 100 cc. of $M/256$ Na_2SO_4 (pH about 6.0) contained 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of $M/4$ KCl , $LiCl$, or $MgCl_2$. The addition of KCl caused only a slight diminution in the rate of diffusion when 12.8 to 25.6 cc. of $M/4$ KCl were added. $LiCl$ caused more of a drop, but a slight addition of $MgCl_2$ caused a considerable drop, as our theory demands.

When, however, a salt with polyvalent anion and monovalent cation like $K_4Fe(CN)_6$ is added to $M/256$ Na_2SO_4 the attraction of the ferrocyanide ion for water counteracts to a large extent the drop caused by the weak repelling action of the K ions added. Fig. 3 shows that the attraction of the $Fe(CN)_6$ ion for water is greater than that of the sulfate ion. Hence the addition of slight quantities of $K_4Fe(CN)_6$ to $M/256$ Na_2SO_4 increases the initial rate of diffusion of water into the solution but as soon as the concentration of $K_4Fe(CN)_6$ added reaches

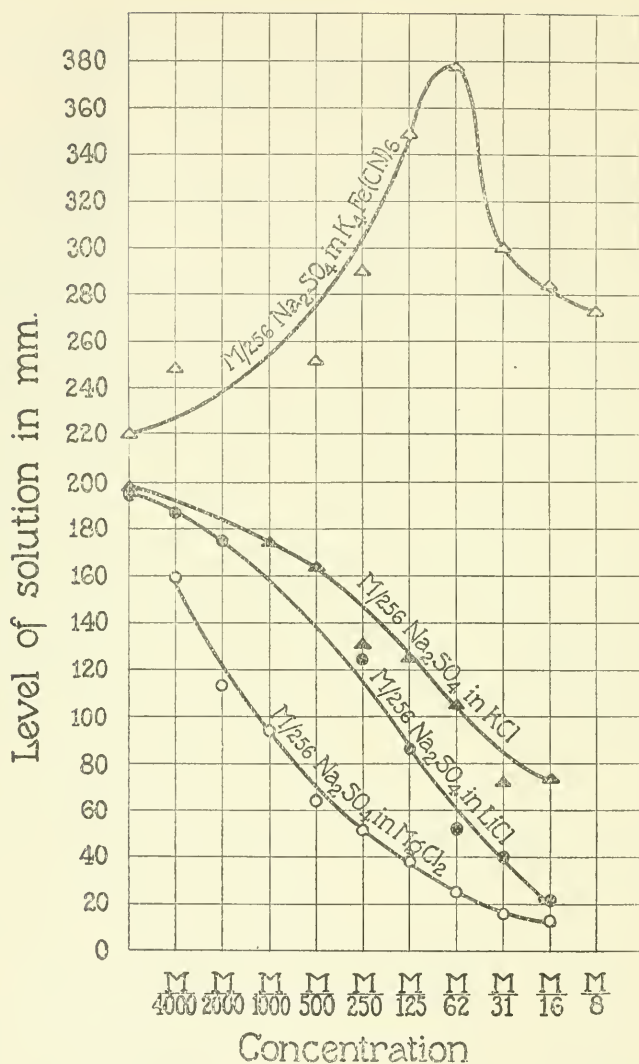


FIG. 14. Curves showing that when we add increasing concentrations of neutral salts with monovalent anion to a neutral $\text{m}/256$ solution of Na_2SO_4 the attraction of the Na_2SO_4 solution for water is diminished. Since this diminution increases in the order K , Li , Mg , the latter being the most depressing ion, it supports the idea that the drop observed in curves of Fig. 3 is due to the repelling action of the cation upon the positively charged particles of water.

the concentration of $M/62$ a further addition of $K_4Fe(CN)_6$ lowers the rate of diffusion of water into the solution. From this concentration on the repelling action of the K and Na ions upon water prevails over the attractive action of the anion.

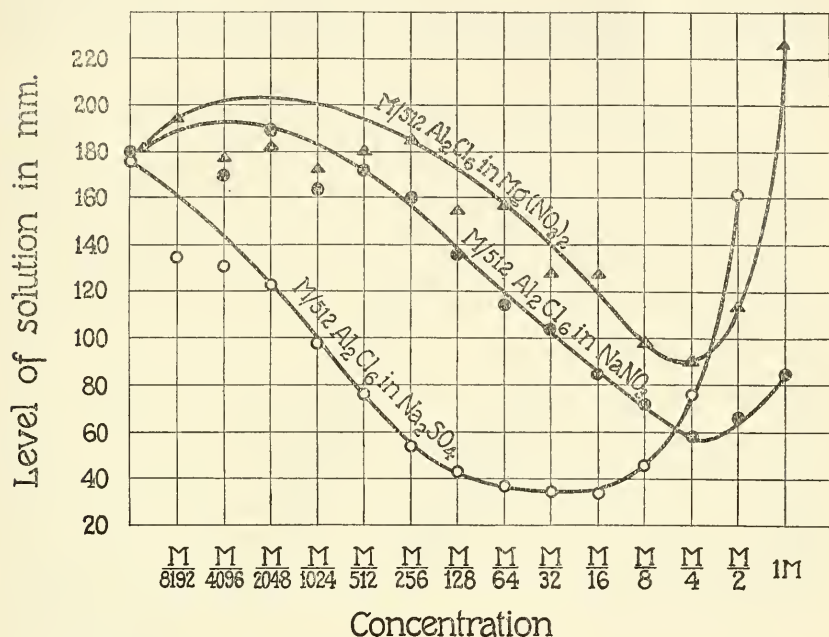


FIG. 15. Curves showing that when we add increasing concentrations of $NaNO_3$, $Mg(NO_3)_2$, and Na_2SO_4 to $M/512$ solutions of Al_2Cl_6 the depressing effect is greater for SO_4 than for NO_3 . Since the water diffuses in this case in the form of negatively charged particles the curves support the idea that the depressing effect of the salt added is due to the repelling action of the anion upon the negatively charged particles of water.

When the collodion flask (previously bathed in gelatin) is filled with $M/512$ Al_2Cl_6 and dipped into a beaker with distilled water, water diffuses at first very rapidly into the flask. The water is in this case negatively charged and attracted by the Al ion and repelled by the anion. When we add small quantities of a salt with bivalent anion, e.g. Na_2SO_4 , to the Al_2Cl_6 solution the attraction of the Al_2Cl_6 solution for water is diminished more rapidly than when we add a salt with a

monovalent anion like NaNO_3 or $\text{Mg}(\text{NO}_3)_2$ (Fig. 15). The Mg ion attracts negatively charged water more powerfully than the NaNO_3 and hence the depressing effect of $\text{Mg}(\text{NO}_3)_2$ is smaller than the depressing effect of NaNO_3 . Fig. 16 shows that the depressing effect of the addition of H_2SO_4 upon $\text{M}/512 \text{ Al}_2\text{Cl}_6$ is greater than the addition of HNO_3 , both acids acting similarly to the potassium salts with the same anion.

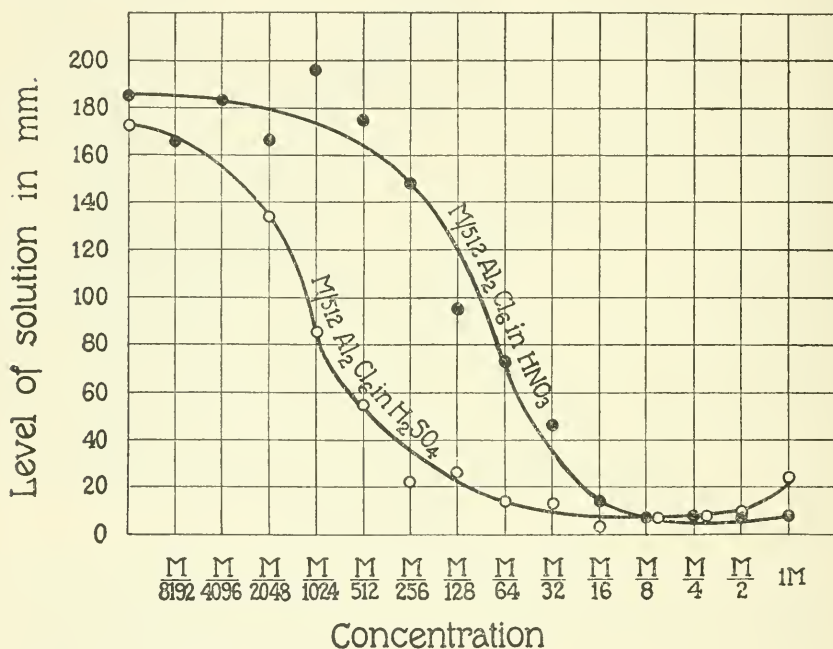


FIG. 16. Curves showing that the depressing effect of HNO_3 and H_2SO_4 is similar to the depressing effect of NaNO_3 and Na_2SO_4 when added to $\text{M}/512 \text{ Al}_2\text{Cl}_6$.

All these facts support our assumption that the drop in the curves representing the influence of the concentration of the electrolyte upon the rate of diffusion of water through collodion membranes is due to the fact that beyond a certain concentration the repelling action of the ion with the same sign of charge as that of water increases more rapidly with a further increase in concentration than the attractive action of the ion with the opposite charge upon the water.

SUMMARY.

1. When a watery solution is separated from pure water by a collodion membrane, the initial rate of diffusion of water into the solution is influenced in an entirely different way by solutions of electrolytes and of non-electrolytes. Solutions of non-electrolytes, *e.g.* sugars, influence the initial rate of diffusion of water through the membrane approximately in direct proportion to their concentration, and this influence begins to show itself under the conditions of our experiments when the concentration of the sugar solution is above $M/64$ or $M/32$. We call this effect of the concentration of the solute on the initial rate of diffusion of water into the solution the gas pressure effect.

2. Solutions of electrolytes show the gas pressure effect upon the initial rate of diffusion also, but it commences at a somewhat higher concentration than $M/64$; namely, at $M/16$ or more (according to the nature of the electrolyte).

3. Solutions of electrolytes of a lower concentration than $M/16$ or $M/8$ have a specific influence on the initial rate of diffusion of water through a collodion membrane from pure solvent into solution which is not found in the case of the solutions of non-electrolytes and which is due to the fact that the particles of water diffuse in this case through the membrane in an electrified condition, the sign of the charge depending upon the nature of the electrolyte in solution, according to two rules given in a preceding paper.¹

4. In these lower concentrations the curves representing the influence of the concentration of the electrolyte on the initial rate of diffusion of water into the solution rise at first steeply with an increase in the concentration, until a maximum is reached at a concentration of $M/256$ or above. A further increase in concentration causes a drop in the curve and this drop increases with a further increase of concentration until that concentration of the solute is reached in which the gas pressure effect begins to prevail; *i.e.*, above $M/16$. Within a range of concentrations between $M/256$ and $M/16$ or more (according to the nature of the electrolyte) we notice the reverse of what we should expect on the basis of van't Hoff's law; namely, that the attraction of a solution of an electrolyte for water diminishes with an increase in concentration.

5. We wish to make no definite assumption concerning the origin of the electrification of water and concerning the mechanism whereby ions influence the rate of diffusion of water particles through collodion membranes from pure solvent to solution. It will facilitate, however, the presentation of our results if it be permitted to present them in terms of attraction and repulsion of the charged particles of water by the ions. With this reservation we may say that in the lowest concentrations attraction of the electrified water particles by the ions with the opposite charge prevails over the repulsion of the electrified water particles by the ions with the same sign of charge as that of the water; while beyond a certain critical concentration the repelling action of the ion with the same sign of charge as that of the water particles upon the latter increases more rapidly with increasing concentration of the solute than the attractive action of the ion with the opposite charge.

6. It is shown that negative osmosis, *i.e.* the diminution of the volume of the solution of acids and of alkalies when separated by collodion membranes from pure water, occurs in the same range of concentrations in which the drop in the curves of neutral salts occurs, and that it is due to the same cause; namely, the repulsion of the electrified particles of water by the ion with the same sign of charge as that of the water. This conclusion is supported by the fact that negative osmosis becomes pronounced when the ion with the same sign of charge as that of the electrified particles of water carries more than one charge.



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THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

VOLUME II, NO. 3

JANUARY 20, 1920



PUBLISHED BIMONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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THE ACTION OF STRYCHNINE AND NICOTINE ON THE
NEUROMUSCULAR MECHANISM OF ASTERIAS.

By A. R. MOORE.

(From the Physiological Laboratory of Rutgers College, New Brunswick, N. J., and
the Marine Biological Laboratory, Woods Hole, Mass.)

(Received for publication, October 23, 1919.)

It has been demonstrated that strychninization in *Asterias* is followed by extreme dorsal flexure of the rays and consequent inability to make righting movements¹ (Figs. 1, 2, and 3). This "spasm" position of the rays is accelerated and accentuated by sensory stimulation. In order to determine the location of the muscles which cause this action, the dorsal myodermal sheath is cut from all five rays of a specimen of *Asterias*. This leaves only the ventral floors of the rays attached to the central disk. When such a preparation is put into a solution of strychnine in sea water, the dorsal flexure of the rays takes place, although not so completely as in the intact animal. This result shows that the ventral walls of the rays contain muscles which act to bend the floor dorsally. The excised pieces of dorsal myodermal sheath when strychninized show dorsal flexure. Therefore, it is evident that in the intact animal, muscles of both the top sheath and floor of the ray act together to flex the ray dorsally.

The reverse picture for the entire animal may be obtained by immersing small specimens of *Asterias* in a solution of alkaloidal nicotine in sea water, concentration 1:50,000. Within a few minutes the rays bend downward at the tips and the central disk is raised (Fig. 4), while at the same time the tube feet are completely shortened and withdrawn. The ventral flexure increases in degree until the animal is a compact mass (Fig. 5). At this point the starfish falls over on its side, where it remains in the knotted condition shown, for 15 or 20 minutes, after which extension takes place.

¹ Moore, A. R., *J. Gen. Physiol.*, 1918-19, i, 97.

Severed rays, when nicotinized, show ventral flexure. Likewise the excised floors of the rays in nicotine solution flex ventrally so far as to form half circles. The isolated top sheaths of the rays, however,

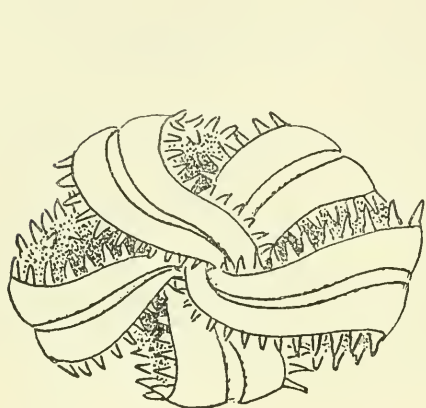


FIG. 1.

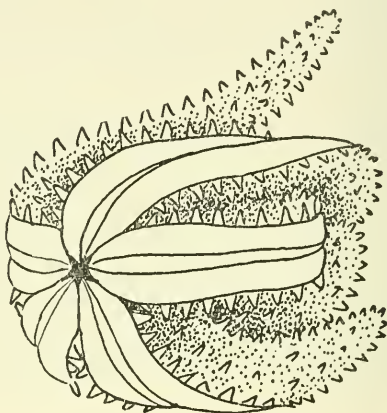


FIG. 2.

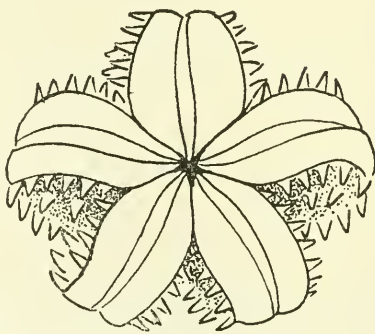


FIG. 3.

FIGS. 1, 2, and 3. Positions taken as the result of strychninization. The arms are bent dorsally to such an extent that they cover up almost completely the back of the animal, a condition illustrated in Figs. 1 and 2. Fig. 3 shows an inverted animal unable to right itself, since all the arms bend dorsally.

when nicotinized, bend dorsally just as if strychninized. From these facts it must be concluded that ventral flexure of the ray depends solely upon certain muscles in the floor of the ray, and that these are

capable of more powerful action than the muscles of the top sheath. It is also clear from the results of strychninization and nicotinization

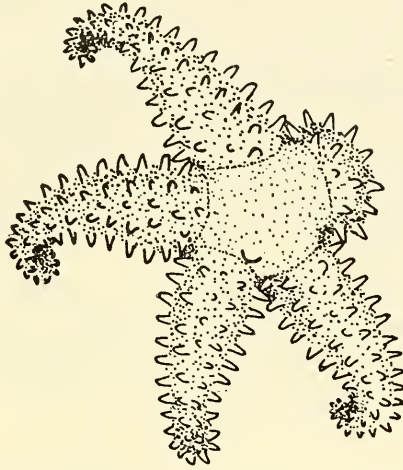


FIG. 4. Initial stage in nicotinization. The effect of the nicotine is the reverse from that of strychnine, since the arms bend toward the ventral side and raise the central disk from the floor.

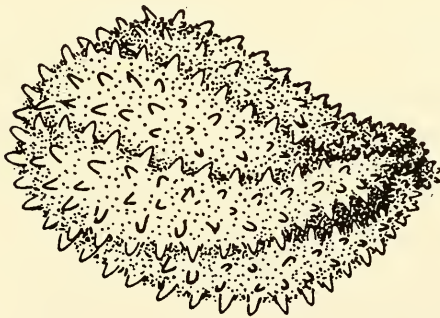


FIG. 5. Final position resulting from nicotinization. The arms are bent towards the ventral side to such an extent that the ventral surface of the animal is no longer seen; the animal falls on its side.

that the ventral floor of the ray contains muscle groups which act antagonistically. If we assume both these drugs to act upon the nervous elements controlling this musculature, we may regard strychn-

nine as an excitor of all such elements. Dorsal flexure then occurs because the sum of the muscles causing it is greater and more powerful than that of the musculature acting ventrally. Nicotine, on the other hand, while exciting the elements of the dorsal sheath, as regards the ray floor, acts specifically to excite the nervous elements controlling the ventral musculature. Nicotine can have no excitatory effect on the innervation of the dorsal antagonists in the ray floor, since this would of necessity result in dorsal flexure of the ray. These facts indicate a difference in the chemical constitution of the two sets of nervous elements concerned with the functioning of the antagonistic muscles of the ray floor.

The preceding analysis is further borne out by the fact that a nicotinized *Asterias* when strychninized shows the characteristic dorsal flexure, while an animal which has been first strychninized cannot be caused to flex ventrally by nicotine.

THE CHROMOSOMES OF PARTHENOGENETIC FROGS.

BY CHARLES L. PARMENTER.

(*From the Zoological Laboratory of the University of Pennsylvania, Philadelphia.*)

(Received for publication, December 1, 1919.)

Among the important features of interest in connection with parthenogenetic frogs are the chromosome number and the sex-determining mechanism. At the suggestion of Doctor Loeb, I have undertaken the investigation of these problems by using some of the parthenogenetic frogs and tadpoles which he has thus far raised.

Previous to 1919, Doctor Loeb had succeeded in raising twenty frogs to the adult condition.¹ Fifteen of these were males, three were females, and the sex of the remaining two was undetermined. In 1919, he succeeded in raising 65 tadpoles to metamorphosis. One of these has metamorphosed, seventeen have been fixed for cytological purposes, five have died, and the rest are still tadpoles.

The chromosomes of the gonads of one of these adult males and of thirteen of the tadpoles have been examined. In all these individuals the number is clearly diploid. The only two spermatogonial complexes of the adult male, sufficiently clear for study, show about twenty chromosomes distinctly and others superimposed, as previously stated.¹ Among the cells undergoing maturation are tetrads in the late prophase stage. These tetrads appear as rings, either completely closed or slightly open at one point. They are apparently of the same form as tetrads of the normal material. Their number is clearly haploid, but an exact count has not yet been obtained. In the sections of the gonads of the thirteen tadpoles there are many complexes in which all but one or two chromosomes are entirely clear, and several mitoses in which all the chromosomes are well separated but cannot be counted with certainty because the cell has been cut in sectioning. However, the number of chromosomes in a limited number of complexes of two individuals is definitely twenty-six.

¹Loeb, J., *Proc. Nat. Acad. Sc.*, 1918, iv, 60.

Since none of the individuals studied has the haploid number, it is probable that the diploid number is characteristic for the majority, if not for all the parthenogenetic individuals. The diploid number, as well as the similarity in form of the tetrads of the parthenogenetic and normal animals, may have been brought about by the retention of the second polar body, or by a premature division of the chromosomes without the division of the cell body just before the first cleavage. It is hoped in the near future to determine how this condition has arisen.

At the present time the mechanism producing the two sexes in both the normal and parthenogenetic frogs is undetermined. Levy² in *Rana esculenta*, and Swingle³ in *Rana pipiens*, describe a sex chromosome in the normal male. But the evidence of neither of these authors is convincing. There are some interesting theoretical possibilities by which a predominance of parthenogenetic males over females, as indicated by the numbers so far obtained, might be produced, and it is hoped that further observations will reveal the exact mechanism.

² Levy, F., *Arch. Mikr. Anat.*, 2te Abt., 1915, lxxxvi, 85.

³ Swingle, W. W., *Biol. Bull.*, 1917, xxxiii, 70.

STUDIES ON BIOLUMINESCENCE.

XII. THE ACTION OF ACID AND OF LIGHT IN THE REDUCTION OF CYPRIDINA OXYLUCIFERIN.

By E. NEWTON HARVEY.

(From the Physiological Laboratory, Princeton University, Princeton.)

(Received for publication, October 17, 1919.)

In the luminous crustacean, *Cypridina*, a substance, luciferin, is oxidized in the presence of water, oxygen, and another substance, luciferase, with the production of light. I have called the oxidation product of luciferin oxyluciferin; it is obtained by allowing luciferin in solution to oxidize completely. The oxidation is accelerated at high temperatures. An extract of *Cypridina*, boiled until it no longer gives light on mixing with luciferase, will be spoken of throughout this paper as an oxyluciferin solution (Harvey, 1918-19). A solution of oxyluciferin has the same reaction as a luciferin solution. It is slightly alkaline, about $\text{pH} = 9$.

No measurable production of CO_2 (Harvey, 1919-20, *a*) or of heat (Harvey, 1919-20, *b*) accompanies the oxidation of luciferin. The reaction, luciferin \rightarrow oxyluciferin, therefore involves a relatively slight energy change and should be readily reversible. An oxyluciferin solution, however, will not reduce to luciferin spontaneously on standing (in the dark) or on removal, with an air pump, of the dissolved oxygen. However, if we add to an oxyluciferin solution some milk, frog muscle suspension, yeast cells, bacteria, or Mg powder plus acid, which results in the evolution of nascent hydrogen, luciferin will be formed and can be tested by its ability to luminesce with luciferase. As the reducing action of milk, tissues, bacteria, and nascent hydrogen are so well known, it seemed probable that they formed luciferin by removal of oxygen from, or addition of H_2 to, oxyluciferin, reducing it in the same way that methylene blue can be reduced (decolorized) by tissue cells, bacteria, nascent hydrogen, etc.

In my previous paper (1918-19) I called attention to the fact that

acid also plays a part in the formation of luciferin from oxyluciferin. "If one saturates an oxyluciferin solution with CO_2 or adds a little dilute acetic acid and allows the solution to stand for 24 hours, a certain amount of reduction will occur" (page 143). Indeed it is not necessary to let the solution stand 24 hours. Luciferin will be formed from an oxyluciferin solution immediately on adding a little acid to it. HCl , HNO_3 , or H_2SO_4 may also be used. The action begins when the solution of oxyluciferin, ordinarily slightly alkaline ($\text{pH} = 9$), is made neutral ($\text{pH} = 7.1$), as indicated in Table I.

TABLE I.
Effect of Acid on Reduction of Oxyluciferin.

Solution.	pH	Luminescence with luciferase.	Remarks.
20 cc. of oxyluciferin alone.	9.01	Negative.	
20 cc. of oxyluciferin + 0.05 cc. of 5 per cent acetic acid.	8.8	"	
20 cc. of oxyluciferin + 0.15 cc. of 5 per cent acetic acid.	7.1	Fair.	
20 cc. of oxyluciferin + 0.03 cc. of 5 per cent acetic acid.	5.9	Good.	Acid forms precipitate in this oxyluciferin solution.
20 cc. of oxyluciferin + 0.05 cc. of 5 per cent acetic acid.		"	Acid forms precipitate in this oxyluciferin solution.
20 cc. of oxyluciferin + 0.75 cc. of 5 per cent acetic acid.		" *	Acid forms precipitate in this oxyluciferin solution.

* Light disappears quickly because of the effect of the acidity on the luciferase.

The acid must act on the oxyluciferin as no luciferase or other enzymes destroyed on boiling are present.

The addition of acid, then, to a boiled extract of *Cypridina* (oxyluciferin) causes the formation of a substance (luciferin) which will give light with luciferase. It is possible that the action of bacteria (which produce CO_2), muscle tissue (which contains lactic acid), or Mg plus acid in forming luciferin is not the result of their reducing power but of their acidity. Fortunately we can test this matter by

the use of reducing fluids which are not acid. If they also form luciferin from oxyluciferin, a reduction must occur. Nascent H can be generated by the action of NaOH on Al or when finely divided Mg, Zn, or Al is placed in water. With Mg the water becomes only slightly alkaline from the formation of almost insoluble $\text{Mg}(\text{OH})_2$. If we add some Al powder and dilute NaOH to an oxyluciferin solution, H is given off and luciferin is formed. As oxyluciferin cannot be formed by the addition of alkali alone we must have in this experiment a reduction of oxyluciferin in alkaline medium by the nascent H produced. Luciferin can also be formed by merely adding Al, Zn, or Mg dust to an oxyluciferin solution. Methylene blue can be readily reduced to its leuco base by Zn dust or Al plus NaOH.

Indeed, if one adds some Al, Zn, or Mg powder to a solution of luciferase, light will appear whenever the solution is shaken. Luciferase solution must always contain the oxidation product of luciferin, oxyluciferin. In the presence of nascent H this is reduced to luciferin and, since the reaction of the medium is alkaline and luciferase is present, this is oxidized with light production, when, by shaking, air is dissolved. The light can never become very bright except at the surface because of the deficiency of oxygen in the solution. It would seem, then, that the action of bacteria, yeast, muscle cells, etc. on oxyluciferin must be due not entirely to their acid reaction but to their reducing power as well.

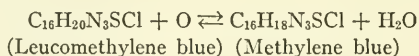
The above experiment is striking and instructive. To a test-tube of luciferase solution containing as it does oxyluciferin add some Zn dust or Mg powder, and the evolution of hydrogen begins. Conditions are now favorable for the reduction of oxyluciferin, and this occurs. Shake the contents of the tube to dissolve oxygen and light appears. Allow the tube to stand and the light soon disappears. Shake again and the light reappears. The reduction and oxidation processes can be demonstrated many times.

A similar experiment can be performed with luciferase plus oxyluciferin solution by the addition of $(\text{NH}_4)_2\text{S}$. This will serve also as another example of the reduction of oxyluciferin in an alkaline medium. Whenever we shake a tube of luciferase, oxyluciferin, and $(\text{NH}_4)_2\text{S}$, light will appear. When the tube is at rest it becomes dark. Even the merest touch is sufficient to agitate the tube con-

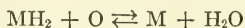
tents, and cause the solution of oxygen and the appearance of light. It is just as if we stimulated the tube to produce light and I believe the phenomenon has a deeper significance and a more fundamental similarity to the phenomena of stimulation than may at first appear. What more simple means of controlling a process can we think of than by admission or withdrawal of oxygen? The firefly turns on its light by stimulation, through nerves, of the luminous organ. *Noctiluca* flashes on stimulation of any kind, even the slightest agitation causing a brilliant emission of light. If the stimulation process means merely the admission of oxygen to the photogenic cells, we have a mechanism in the cell itself for automatically producing the light. The admission of oxygen results in aerobic conditions, and luciferin in the presence of luciferase can then oxidize to oxyluciferin with luminescence. When the oxygen is used up, the light ceases, anaerobic conditions prevail, and the oxyluciferin is reduced to luciferin again. Thus, luciferin is reformed during the rest period of *Noctiluca* or between the flashes of the firefly. What more efficient type of light than this is to be desired.

Again, methylene blue offers an interesting parallel to oxyluciferin. A little $(\text{NH}_4)_2\text{S}$ added to a methylene blue solution will reduce (decolorize) it to the leuco base. If the tube is now shaken, the blue color returns. On standing reduction again occurs. The process can be repeated a number of times, the reaction going in one or the other direction depending on the oxygen content of the mixture.

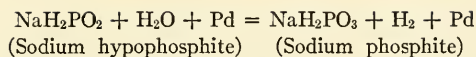
As methylene blue contains no oxygen its reduction consists in the addition of two atoms of hydrogen. When leucomethylene blue oxidizes, water is formed by the union of these two atoms of hydrogen with oxygen, thus



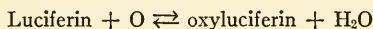
More briefly



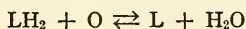
To reduce methylene blue we can add the two hydrogen atoms directly from nascent hydrogen formed in the solution or we can split up water by a catalyzer in the presence of some substance which will take up the oxygen of water. The hydrogen of water then adds to the methylene blue, thus



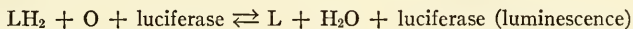
This reaction occurs in the presence of finely divided palladium. The methylene blue is reduced by the H_2 , and the hypophosphite is oxidized. Since oxyluciferin can be reduced by palladium and sodium hypophosphite (Harvey 1918-19), it is probable that we can write the equation for the reduction of oxyluciferin and oxidation of luciferin in a similar manner to that of methylene blue.



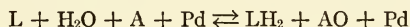
More briefly



Just as in the case of methylene blue the reaction proceeds in the right hand direction spontaneously if the pressure of O is sufficiently high. If luciferase is also present we have luminescence.



The reaction proceeds in the left hand direction under low oxygen pressure, in the presence of nascent hydrogen, or with some catalyzer which is able to split water, transferring the H_2 to oxyluciferin and the O to an acceptor (A). NaH_2PO_2 plays the part of the acceptor.



This appears to be the way in which the reducing enzymes or perhydridases (comparable to the Pd) of living tissues act (Bach, 1911, 1912, 1913), and the action of yeast cells, bacteria, muscle suspensions, etc. in reducing oxyluciferin must occur in the same manner.

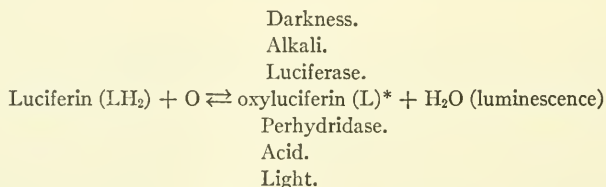
If we assume that the LH_2 (luciferin) compound is dissociated even to the slightest extent into L and hydrogen, the hydrogen ion will shift the equilibrium toward the formation of that substance which involves the taking up of hydrogen. Consequently we may obtain a partial formation of luciferin by adding an acid to oxyluciferin. Reduction of the hydrogen ion concentration tends to shift the equilibrium in the opposite direction. Consequently, addition of alkali favors the oxidation of luciferin and it is quite generally true that biological oxidations are favored by an alkaline reaction. In addi-

tion oxygen in alkaline medium has a higher oxidation potential than in neutral or acid media. I believe this is the explanation of the action of acid in the formation of luciferin from oxyluciferin.

Addition of acid is not the only means of favoring the formation of luciferin from oxyluciferin. Any reaction which proceeds in one direction with evolution of light should, theoretically, proceed in the opposite direction under the influence of light. As far as I know the case of a reaction, photogenic in one direction and photochemical in the other direction, has never been described, unless we are to accept the cases of phosphorescence; for instance, the absorption of light by CaS and its emission in the dark. However, the reaction which occurs during phosphorescence cannot be stated.

It is a fact that light will cause the reduction of oxyluciferin. A tube of oxyluciferin exposed to sunlight for 6 hours or the mercury arc for 2 hours will be partially converted into luciferin. It will luminesce when luciferase is added while a control tube kept in darkness shows no trace of luciferin. The action is more marked with the ultra-violet, as a solution of oxyluciferin in a quartz tube showed more reduction than one in a glass tube when exposed for the same length of time to the quartz mercury arc. The reduction is not dependent on the formation of acid under the influence of light since two tubes of oxyluciferin, one kept in darkness and the other exposed to sunlight for 6 hours, had the same reaction, pH = 9.3. Of course some reducing substance might be formed under the influence of light but this is not very probable.

We may therefore write the reaction for luminescence in the following way.



* It is unfortunate that Dubois (1918) has used the word oxyluciferin in quite a different sense from my own. His idea of the processes involved is as follows (p. 319):

As I stated in my previous paper (Harvey, 1918-19, page 144), "Acid thus favors reduction and hinders oxidation, while alkali favors oxidation and hinders reduction" so we may now add, light favors reduction and darkness favors oxidation of the luciferin \rightleftharpoons oxyluciferin reaction.

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 Harvey, E. N., *J. Gen. Physiol.*, 1918-19, i, 133; 1919-20, ii, a, 133; b, 137.
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"Coluciférase + pré-luciférine = luciférine;
 Luciférase + luciférine = oxyluciférine;
 Oxyluciférine + oxygène = lumière."

Coluciferase forms luciferin from a precursor, preluciferine, and Byla's peptone, lecithin, esculin, taurine, tyrosine, and asparagine will also form luciferin if mixed with coluciferase. We may perhaps add that luciferin must have an extraordinary chemical structure, if it can be formed from such diverse substances as lecithin, esculin, and taurine. My own view of the processes involved in luminescence is given in the text.

In a later paper, appearing after my own (Harvey, 1918-19), Dubois (1919) makes no mention of preluciferine and accepts in the case of *Pholas* my view that luciferin can be again formed from a boiled extract of luminous animals by reduction. The enzyme of *Pholas* concerned in this reduction ("a hydrogenase") can also liberate hydrogen from cane sugar. As this is Dubois's first mention of luciferin formation by reduction, I fail to see how, as Dubois states, I "rediscovered" this fact for *Cypridina*.

STUDIES ON ENZYME ACTION.

XVIII. THE SACCHAROGENIC ACTIONS OF POTATO JUICE.

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(Received for publication, November 14, 1919.)

INTRODUCTION.

The results of enzyme studies on a number of fresh and dehydrated vegetables were communicated in a previous paper.¹ In connection with the experiments on amylase, it was there stated that: "Potato juice, when tested for amylase by the method used with the other vegetable juices, gave an end-point which was so uncertain and difficult to determine that a different method had to be employed for it." In this paper are presented the results of a more careful study of potato amylase. Evidence is given showing the occurrence of a sucrase, and of a certain amount of sucrose or raffinose in potato juice.

Experimental Method.

An extended study of potato amylase was published by Doby.² Attempts were made to repeat his work, but it was soon found that his observations were incomplete. In his work a series of tubes containing varying amounts of potato juice and Lintner soluble starch were set up for each experiment. After incubation, the supernatant liquid in each tube was pipetted from a residue, which separated as a rule, and was tested with iodine. The measure of the action was given by the tube in which no starch test was given with the iodine. Doby stated that the residue or precipitate contained no starch, but he did not present any experiments of his own in this connection. This statement apparently is based upon the work of

¹ Falk, K. G., McGuire, G., and Blount, E., *J. Biol. Chem.*, 1919, xxxviii, 229.

² Doby, G., *Biochem. Z.*, 1914, lxvii, 166.

other investigators,³ who, however, worked with animal liquids, and not with filtered vegetable juices.

Upon repeating these experiments, it was found that, in accordance with the results of Doby, the supernatant liquids in many cases gave no test for starch, but on testing the precipitates with iodine, blue colors or compounds were obtained, showing the presence of starch. The presence of starch could also be shown by testing the whole mixture with iodine and shaking. A blue or purple color would be formed throughout the mixture, which would settle with the precipitate, leaving the supernatant liquid colorless. Wherever, therefore, a precipitate was formed, the results of the test as given by Doby must be looked upon as inconclusive. He found that sodium fluoride accelerated the amylase action. This observation can evidently be interpreted as the formation of a precipitate of the starch with the amylase, protein, or other material present which was produced more readily in the presence of this salt. With larger amounts of potato juice, hydrolysis of the starch would be complete, but the whole mixture must be tested in every case. These conditions were not realized by Doby in many of his experiments.

A different difficulty was encountered in attempting to use the Wohlgemuth⁴ method for following amylase actions. In most of the experiments, in testing the final mixture or even the initial solution with iodine, an intense blue or purple color was obtained, which, however, faded rapidly, sometimes in a few seconds, leaving the mixture colorless. The rapidity of fading depended upon the quantity of potato juice present. It was necessary to add considerable iodine solution before a color, constant even for 3 or 4 minutes, could be obtained. Also, the end-points of these solutions were uncertain. Even when constant colors were obtained, these ranged from yellow-gray through gray to blue so that it was difficult to decide which tube represented the complete or final decomposition of the starch. Because of the presence of the chemically unknown, iodine-combining components of potato juice and the uncertainty of determining the

³ Starkenstein, E., *Biochem. Z.*, 1910, xxiv, 191. Schirokauer, H., and Wilenko, G. G., *Biochem. Z.*, 1911, xxxiii, 275.

⁴ Wohlgemuth, J., *Biochem. Z.*, 1908, ix, 1.

end-point of the actions, it was decided not to use this method in this work.

The Lintner⁵ method, which was used with the vegetable juices for which the results were given in the former paper,¹ could not be used because the cuprous oxide was formed in such a finely divided state that it was impossible to tell whether the remaining Fehling's solution was still blue or had become colorless.

The method developed by Sherman, Kendall, and Clark,⁶ precipitating and weighing the cuprous oxide from an excess of Fehling's solution under definite conditions, was finally adopted.

The general method of experimentation was as follows: The potatoes were thinly peeled, weighed, ground in a food chopper, squeezed through muslin, then the juice was filtered through fresh muslin, and finally filtered through asbestos on a Buchner funnel. The insoluble starch was removed as completely as possible in this way, but the solutions still contained a certain amount of soluble starch. The actions of the enzymes on the soluble starch and simpler carbohydrates present in the solutions, as well as on added starch and other substances, were followed. The tests were set up with 25 cc. of potato juice and 50 cc. of 1 per cent Lintner soluble starch solution, 50 cc. of water, or 50 cc. of 1 per cent cane sugar or maltose solution, as desired. Toluene was added as a preservative. Immediately after mixing, 5 to 15 cc. were pipetted out for the determination of the reducing action for blanks. The remainder was incubated for 18 to 24 hours at 37–40°C., and portions were then removed for tests. For the determination of the reducing action, Sherman's method was followed closely. The portion taken, 5 to 15 cc., was diluted in an Erlenmeyer flask with boiling water to 50 cc., 50 cc. of Fehling's solution were added, heated in a boiling water bath for 15 minutes, filtered on an asbestos mat in a porcelain Gooch crucible, washed with boiling water, alcohol, and ether, dried at 100°, and weighed. The cuprous oxide was then dissolved and the crucibles reweighed to check up possible mechanical losses of the mats. The starch solution was prepared by adding a paste of the Lintner soluble

⁵ Lintner, C. J., *J. prakt. Chem.*, 1886, xxxiv, 378.

⁶ Sherman, H. C., Kendall, E. C., and Clark, E. D., *J. Am. Chem. Soc.*, 1910, xxxii, 1083.

starch in water to the requisite amount of boiling water, boiling for 1 to 2 minutes, and cooling rapidly. The added sugars which were used (sucrose and maltose) were the purest obtainable. The results were calculated to correspond to 15 cc. of the original mixture, except where stated otherwise, and are given in terms of mg. of cuprous oxide obtained.

Sherman and Schlesinger⁷ compared the amyloclastic and saccharogenic actions of certain amylases, and pointed out clearly the significance of these actions. In this paper, the saccharogenic actions of the enzymes in potato juice, as determined by the amounts of reduced products, are followed and not the amyloclastic (splitting of starch into products which do not give a blue color with iodine). While the two ways of determining the actions undoubtedly overlap to a certain extent, the differences must also be borne in mind. In any event, the chemical changes in either case are not known except with the disaccharides used, so that a strictly quantitative comparison of the actions is not so certain as in other enzyme actions. The possibility of a small amount of cuprous oxide being held in solution by other substances present in the juices must also be considered. Attempts were made to eliminate this and other sources of error by running blanks wherever possible.

The solutions were brought to definite hydrogen ion concentrations before mixing by the addition of sodium hydroxide or hydrochloric acid solutions of various concentrations and tested with the indicators recommended by Clark and Lubs.⁸

Experimental Results.

The properties of the juices from the different lots of potatoes varied somewhat. In general terms, the specific gravities of the juices from new and winter potatoes ranged between 1.025 and 1.035, those from sprouted potatoes (only small, shriveled ones were obtainable) somewhat higher, from 1.035 to 1.040. Almost twice as much juice was obtained from the former as from the latter, approximately 20 to 30 per cent by weight of the original solid with

⁷ Sherman, H. C., and Schlesinger, M. D., *J. Am. Chem. Soc.*, 1913, xxxv, 1784.

⁸ Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 109, 191.

the former. The residues after removal of the juice showed moisture contents of between 60 and 70 per cent when dried at 100°. The juice as obtained contained on an average between 3 and 4 mg. of nitrogen per cc. for new potatoes, and somewhat more, 4 to 5 mg., for winter and sprouted potatoes.

The hydrogen ion concentration of the juice as prepared was very close to 10^{-6} N or pH 6.0 in every case.⁹

Although the properties of the juice from different lots of potatoes varied somewhat, an attempt was made to make each experiment as complete as possible by running all the necessary blanks. The actions found, therefore, should have general validity. It may also be stated that duplicate determinations agreed satisfactorily.

The results of the actions of the enzymes in the juice upon the constituents of the juice itself and upon added soluble starch solution are given in Table I. The action on the added starch is taken as the difference between the action obtained on the juice plus water and that of the juice plus starch. The results are given as mg. of cuprous oxide obtained by the reducing actions of 15 cc. portions of the solutions. The juice itself was found to have a pH value of 6.0, and the solutions of different acidities were made up by the addition of small quantities of different strengths of acid or alkali. This prevented undue dilution and made the results practically comparable without further calculation. The values of the reductions obtained were corrected for blanks, so that the table shows the actual amount of Cu_2O corresponding to the reducing substances formed during incubation due to the enzyme actions. For the fresh juice the blanks ranged in the extreme cases between 100 and 200 mg. of Cu_2O . The enzyme actions did not appear to depend directly on the reducing actions of the juice although it seemed in a number of experiments as if, as with the cabbage, carrot, yellow turnip, and white turnip amylases, the greater the blanks (on the fresh juice) the greater were the actions.

There were considerable variations in the individual experiments, but the large number of determinations made should make it possible to obtain satisfactory mean values. The figures in parentheses in Table I give the mean values for the separate preceding figures.

⁹ Cohn, E. J., Gross, J., and Johnson, O. C., *J. Gen. Physiol.*, 1919-20, ii, 145.

TABLE I.
Saccharogenic Actions of Potato Juice.

pH	New potatoes.		Winter potatoes.		Sprouted potatoes.	
	Change in juice.	Action on added starch.	Change in juice.	Action on added starch.	Change in juice.	Action on added starch.
2	96.6	27.5				
	72.0 (84.3)	(27.5)				
3	23.0	3.1				
	59.8	7.7				
	30.7					
	(37.8)	(5.4)				
4	12.4	0.6	11.5	0	16.6	0
	23.0	6.1	6.5	0		
	10.0	0	26.9	0		
	10.5	3.9	0	0		
	12.5	0.7	9.0			
	14.4	0				
	16.1	0				
	18.6	0				
	14.8	0				
	(14.7)	(1.3)	(10.8)	(0)	(16.6)	(0)
5	3.6	10.9	46.3	0	31.6	3.3
	8.7	7.3				
	9.9	2.7				
	16.2	1.8				
	9.0	16.4				
	(9.5)	(7.8)	(46.3)	(0)	(31.6)	(3.3)
6	36.3	20.0	37.4	26.3	44.5	39.1
	16.0	27.2	49.4	28.0		
	32.7	26.3	28.7	35.3		
	5.7	23.8	45.5	33.0		
	6.1	31.4	44.4	32.5		
	10.7	38.2				
	14.5	11.4				
	12.5	28.8				
	18.6	26.3				
	(17.0)	(25.9)	(41.1)	(31.0)	(44.5)	(39.1)
7	18.2	15.4	30.4	17.5	23.0	39.6
	20.7	36.6				
	22.8	25.1				
	(20.6)	(25.7)	(30.4)	(17.5)	(23.0)	(39.6)

TABLE I—*Concluded.*

pH	New potatoes.		Winter potatoes.		Sprouted potatoes.	
	Change in juice.	Action on added starch.	Change in juice.	Action on added starch.	Change in juice.	Action on added starch.
8	6.4	26.0	7.0 14.1	7.7 0.4	8.1	34.6
	0	22.0				
	0	6.3				
	0	8.0				
	4.8	9.3				
	3.6	14.7				
	13.1	16.1				
	(4.0)	(14.6)				
9	10.1	2.2	1.9 (1.9)	6.6 (6.6)	(8.1)	(34.6)
	4.4	6.3				
	0	3.9				
	(4.8)	(4.1)				
10	0	0				
	(0)	(0)				

Table II shows the ratios of the changes in the juice to the actions on the added starch, with the latter taken as unity, at the different hydrogen ion concentrations.

TABLE II.

Ratios of Changes in Juice to Actions on Added Starch (as Unity).

pH	New potatoes.	Winter potatoes.	Sprouted potatoes.
2	3.1		
3	7.0		
4	11.3	∞	∞
5	1.2	∞	9.6
6	0.66	1.3	1.1
7	0.80	1.7	0.58
8	0.27	(2.6)	0.23

In addition to the results shown in Tables I and II, some work may be mentioned without entering into detail. In order to determine whether an excess of substrate was present in the juice itself,

a number of samples were hydrolyzed completely with dilute acid. The results showed that for 15 cc. portions in which the blanks (original reducing actions) gave 120 to 150 mg. of Cu_2O , reduction after hydrolysis gave 220 to 250 mg. of Cu_2O . An excess of substrate capable of forming reducing substances upon hydrolysis was therefore present.

In general terms, the juice from winter potatoes showed greater blanks and somewhat greater actions than the juice from new potatoes, and that from sprouted potatoes greater than that from winter potatoes. These conclusions were confirmed by several experiments with new potatoes which were "aged" for from 1 to 2 weeks in a warm room. The juice showed somewhat greater blanks and greater actions after this "aging" of the potatoes.

Doby² had found by his method that sodium fluoride accelerated the action of potato juice on added starch. In attempting to repeat this work, by testing the residue, not merely the supernatant liquid, with iodine, it was found that the action was not accelerated as he stated. Further, by using the reduction method, it was found that the action was retarded by the sodium fluoride at the indicated concentration (0.3 per cent). As pointed out before, the conclusions of Doby were based upon incomplete observations.

Discussion of Results.

The results of Tables I and II may be summarized briefly. In view of the greater number of experiments with new potatoes, the conclusions will be based on the results with these, the other series being used as confirmatory.

With the juice alone, a large action was observed in the acid solution (pH 2), decreasing as the acidity decreased to pH 5, then increasing again, showing a maximum at pH 6 to 7. The actions then decreased with increasing alkalinity, becoming 0 at pH 9 to 10.

The action of the juice on added soluble starch was marked at pH 2, although less than on the juice constituents themselves. This action decreased with decreasing acidity, giving a minimum at about pH 4, increased again with a maximum at pH 6 to 7, and then decreased to 0 at pH 9 to 10.

The two series of actions, on the juice constituents and on the added starch, show definite differences which are also brought out in Table II.

In the first place, it is interesting to note that both sets of actions show maxima at pH 6 to 7, approximately the acidity of the naturally occurring material. This was also observed with the amylase actions of the vegetable juices reported in the previous paper.

In comparing the two sets of actions, at pH 4, the action on the juice constituents is much greater than on the added starch; approximately the same at pH 6 to 7; and reversing at pH 8 where the action on the added starch is much greater than on the juice constituents themselves.

The increase in reducing substances of the juice alone and the juice with added starch is not, in all probability, a simple action. The nature and amounts of the possible products formed were not investigated, so that an accurate quantitative comparison is not feasible. The action on added starch, showing a maximum at pH 6 to 7, may be compared to maxima for the juices of the cabbage, carrot, and white turnip at about pH 6, for yellow turnip, ranging from pH 4 to 7; for pancreatic amylase,¹⁰ pH 7; for malt amylase,¹⁰ pH 4.4 to 4.5; and for *Aspergillus oryzae* amylase,¹⁰ pH 4.8. This action of potato juice on added starch is without doubt an amyloclastic action. The increase in reducing action of the juice alone may, however, be of more complex nature. The results shown in Tables I and II indicate the possibility that, in addition to the amylase acting upon the starch of the juice, other enzymes may be acting upon other constituents. This view was tested experimentally, and it was found that at least a sucrase and also either sucrose or raffinose or both were present, and that presumably the sucrase hydrolyzed the sucrose or raffinose. No evidence of the presence of a maltase was obtained.

Sucrase and Maltase Experiments.

In order to test for the presence of a sucrase in potato juice, series of experiments similar to those described were set up including additional series containing enough sucrose to make 1 per cent solutions.

¹⁰ Sherman, H. C., Thomas, A. W., and Baldwin, M. E., *J. Am. Chem. Soc.*, 1919, xli, 231.

The mean results of three such series, corrected for blanks, each at pH 4, 6, and 8, are given in Table III.

TABLE III.

Actions of Potato Juice in Presence and Absence of Sucrose.

pH	No sucrose added.		Sucrose added.		Difference due to sucrose.	
	Change in juice.	Action on added starch.	Change in juice.	Action on added starch.	Change in juice.	Action on added starch.
4	12.2	0	38.7	0	26.5	0
6	8.1	28.0	12.0	27.5	3.9	0
8	1.8	11.4	6.1	12.7	4.3	1.3

The results in the last two columns show the presence of a sucrose-hydrolyzing enzyme much more active in the slightly acid (pH 4) solution when acting upon potato juice plus sucrose.

The presence of a maltase in potato juice was tested for in a similar way by using 1 per cent maltose solutions in place of 1 per cent sucrose solutions. The results are given in Table IV.

TABLE IV.

Actions of Potato Juice in Presence and Absence of Maltose.

pH	No maltose added.		Maltose added.		Difference due to maltose.	
	Change in juice.	Action on added starch.	Change in juice.	Action on added starch.	Change in juice.	Action on added starch.
4	12.2	0	6.1	1.2	-6.1	-1.2
6	12.6	27.9	14.5	10.5	1.9	-17.4
8	1.2	9.5	1.1	3.3	-0.1	-6.2

No maltase is present in the juice as prepared as far as can be told from these results. The one small positive action obtained on the juice appears to be within the limits of experimental error. The added maltose acts in fact to retard the actions, especially markedly in connection with the action on the added starch. Because of the complexity of the reactions involved in the hydrolysis of starch, this does not appear to be a mass action effect of the products of the reaction retarding further action, but can better be referred to an action between maltose and the amylase, possibly a chemical combi-

nation by which the amylase is prevented from reacting with the starch.¹¹

Since the existence of a sucrase was demonstrated in potato juice, it was of interest to determine whether a carbohydrate upon which this enzyme would act was present. As far as known, sucrase acts only upon the carbohydrates, sucrose and raffinose.

The sucrase solution¹² had been prepared from yeast, and the extraneous matter removed by the method of Nelson and Born.¹³ This solution exerted no hydrolyzing action on soluble starch or maltose. In order to test the action on potato juice without the interference of the enzymes present in the juice, the latter was heated for 15 minutes in a boiling water bath after the temperature of the juice had reached 80°C. The final temperature was about 90°C. This inactivated the enzymes. The juice was then filtered through paper. It was assumed that the sucrose or raffinose, if present, would not be destroyed and would remain in solution. That some change, presumably partial hydrolysis of some of the starchy materials present, did take place, was shown by the increased reduction (20 to 50 per cent) of the blanks.

In carrying out the tests, the juices as prepared were brought to the requisite hydrogen ion concentrations and 2 cc. of a sucrase solution, made by diluting 1 cc. of the filtered stock solution to 500 cc., were added. 0.5 and 1.0 per cent sucrose solutions similarly treated were run simultaneously. Suitable blanks were also run in every case. After incubation for 20 to 24 hours with toluene as a preservative, the actions were determined by the reduction of Fehling's solution by 5 cc. of the solutions as described above. The results are shown in Table V and are given in terms of mg. of Cu₂O obtained by the reduction by 5 cc. portions, corrected for all blanks.

Each row represents a separate series of experiments. The differences in the sucrose results are due to deterioration of the sucrase solution which stood in a warm room for 3 months. The potato juice results show satisfactory agreement in view of the differences in

¹¹ Wohl, A., and Glimm, E., *Biochem. Z.*, 1910, xxvii, 349.

¹² A solution of sucrase was supplied by Professor J. M. Nelson of Columbia University for the tests for the presence of sucrose or raffinose.

¹³ Nelson, J. M., and Born, S., *J. Am. Chem. Soc.*, 1914, xxxvi, 393.

TABLE V.

Actions of Yeast Sucrase on Boiled, Filtered, Potato Juice Solutions.

	pH				
	4	5	6	7	8
0.5 per cent sucrose.	8.6	9.3			
1.0 " " "	(6.6)	11.5	12.2	2.2	0
Boiled filtered juice.	14.0	15.6			
	12.5	9.0	9.1	4.9	1.8
	12.1	10.8	9.6	(8.8)	1.2
	8.6	11.3	7.9		
			7.8	3.9	1.2

the juices as prepared. The two results in parentheses are probably incorrect.

The results, compared with the results on sucrose, show that the two sets of actions are similar. Optimum actions were obtained over approximately the same range of acidity. This proves that sucrose (or raffinose) is present in the juice, and that its concentration is of the order of magnitude of 1 per cent.¹⁴

CONCLUSIONS.

From the actions observed with potato juice alone and in the presence of added starch, it is now possible to state definite conclusions. The marked actions at pH 4 to 5 of the juice alone and also in the presence of the added starch were due to the hydrolysis of sucrose (or raffinose) by the sucrase of the juice. There was very little action upon the starch of the juice itself or upon added starch. At pH 6, the action observed in the juice alone was made up of the action of the sucrase and of the amylase, both on the constituents of the juice; additional action was observed on the added starch. At pH 7 to 8, there was little action of the sucrase, only that of the amylase being observable.

The amylase had an optimum pH of 6 to 7 and acted in the same way upon both the starch occurring in the juice and upon the added

¹⁴ König, J., *Chemie der menschlichen Nahrungs- und Genussmittel*, Berlin, 4th edition, 1904, 895, 897.

starch. The marked action observed in the more acid solutions was due to the sucrase.

SUMMARY.

The saccharogenic enzymes present in potato juice were studied. The actions were followed upon the substances present in the juice and upon added sucrose, maltose, and soluble starch.

Sucrase and amylase were found to be present in the juice. No indication of a maltase was obtained.

The sucrase showed optimum conditions for action at pH 4 to 5, the amylase at pH 6 to 7, both upon the starch present in the juice and upon added soluble starch.

The action of a yeast sucrase preparation upon the juice showed the presence of sucrose (or raffinose) in a concentration of the order of magnitude of 1 per cent.

THE PHOTOCHEMICAL NATURE OF THE PHOTSENSORY PROCESS.

By SELIG HECHT.

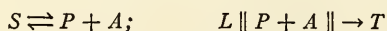
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(Received for publication, November 21, 1919.)

I.

Many animals which are sensitive to light respond by an invariable, characteristic reflex. Typical of such organisms are the ascidian, *Ciona intestinalis*, and the common, North Atlantic, long-neck clam, *Mya arenaria* (Fig. 1). Both of these animals, when illuminated, respond by a vigorous retraction of the siphons. The properties of their sensitivity have been investigated to some extent, and have been described in a series of papers (Hecht, 1918-19, *a, b, c, d*). As a result, an hypothesis has been suggested which accounts for this type of irritability in terms of an underlying, chemical mechanism.

In its essentials this hypothesis involves the behavior of two processes: one, a reversible photochemical reaction; the other, an ordinary, simple, chemical reaction. The light acts on a photosensitive substance *S* and decomposes it into its two precursors *P* and *A*. The degree of sensitivity of the sense organ depends, not on the quantity of photosensitive substance *S*, but on the concentration of its precursors *P* and *A*. Because of this, the amount of fresh precursors necessary for a response is always a constant fraction of the amount of precursors already present in the system. The fresh precursors serve to catalyze the simple, chemical conversion of an inactive substance *L* into one *T* which then initiates the nervous impulse. This eventually leads to a contraction of the siphon musculature. The reaction system as a whole may be expressed in the following equations:



in which $\parallel P + A \parallel$ means catalysis by *P* or *A*, or both.

The evidence which has already been published has established different phases of this hypothesis. (1) Dark adaptation depends on the regular decrease in the concentration of the residual precursors present in the sensory system. This disappearance of precursors is independent of light, proceeds according to the dynamics of a bimolecular reaction (Hecht, 1918-19, *b*), and most probably results in the reformation of photosensitive material. (2) This "dark" reaction has a temperature coefficient of 2.4 for 10°C., similar to those usually found for chemical processes. (3) It is quite characteristic of catalyzed reactions that the velocity of the reaction is a linear function of the concentration of catalyst. Similarly we find that the velocity of the second reaction, $L \rightarrow T$, is a linear function of the concentration of freshly formed precursor catalysts (Hecht, 1918-19, *c*). (4) The simple chemical nature of the catalyzed reaction, $L \rightarrow T$, is evidenced by its quantitative behavior in relation to the temperature. It follows the theoretical expectation according to the Arrhenius equation

$$k_1 = k_0 e^{\frac{\mu}{2} \left(\frac{1}{T_0} - \frac{1}{T_1} \right)}$$

in which the velocity constants (k) vary with the absolute temperatures (T). In this instance the constant $\mu = 19,680$, a value characteristic of simple processes like hydrolyses and saponifications (Hecht, 1918-19, *d*).

In spite of this array of evidence, there is one significant portion of the hypothetical chemical system, the validity of which still remains to be demonstrated. This is the assumption that the reaction $S \rightarrow P + A$ is really photochemical in nature. In other words, it has still to be proved that the action of the light on the sensory process possesses the ordinarily well demonstrated characteristics of photochemical reactions. The present investigation has therefore been concerned with precisely this aspect of the matter; and it is the object of this paper to show that the initial effect of the stimulus is indeed photochemical in nature.

II.

The experiments were performed with the clam, *Mya arenaria*, which is so common at Woods Hole, Mass. An idea of the appearance of this animal may be obtained from Fig. 1, which is made from

the photograph of a living, medium sized individual expanded in sea water. The extent to which the photosensitive siphon may be protruded is variable; the animal in the figure represents an average condition. On stimulation the siphon is shortened after a clearly defined reaction time. The response is well marked, and involves a movement of the tip of the siphon toward the shell. The amount of this retraction is about 1 cm., often it is more, and it is rarely less than $\frac{1}{2}$ cm. There is never any doubt about the occurrence of a response, nor of the exact moment when the retraction begins. The reaction time may therefore be measured with considerable accuracy.

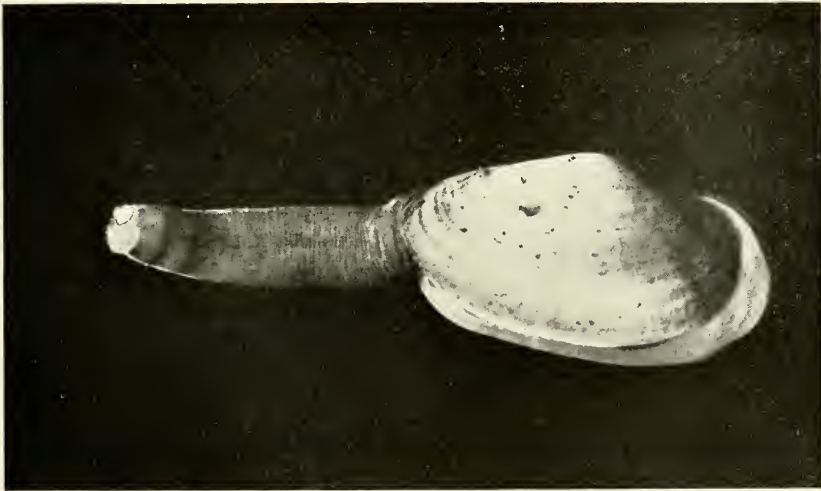


FIG. 1. From a photograph of a medium sized, living individual of *Mya arenaria*, expanded in sea water. The reproduction is a little less than life size.

The reaction time is not a simple interval. It consists of two distinct periods. The first is the exposure or sensitization period. This is very short, and is the time occupied by the actually necessary exposure to light. The bulk of the reaction time is composed of the second phase, the latent period. During this period it is not necessary for the siphon to be illuminated. Thus an animal which has been exposed to a flash of light of a few hundredths of a second duration will respond in approximately 2 seconds, even though at that moment it is in the dark.

In order to demonstrate graphically this division of the reaction time, I used an optical recording device.¹ The animal is placed vertically in sand in a glass trough filled with sea water. A fine thread connects its siphon tip with a light heart lever as near the axle as possible. To the opposite side of the axle is attached a small mirror which reflects a beam of light into the slit of the recording camera.

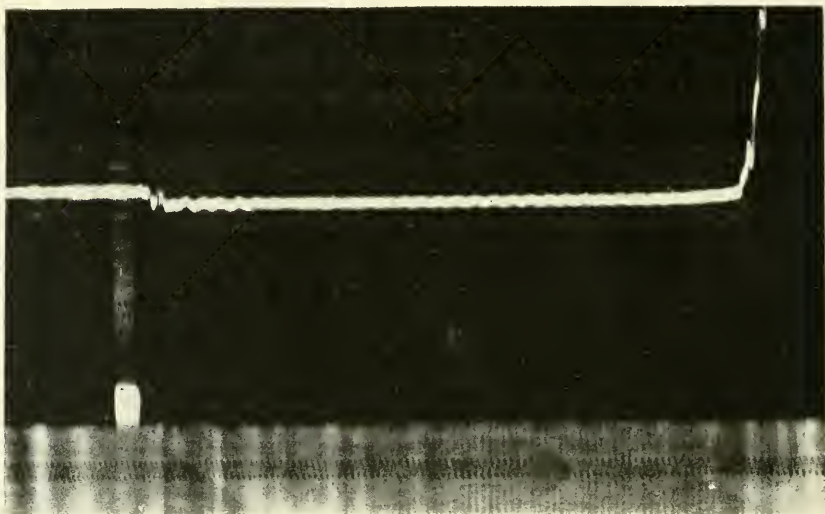


FIG. 2. Optical record of stimulation and response, showing the short exposure period and the long latent period. The white streak running the length of the figure represents the movement of a mirror attached to the siphon tip of *Mya*. The white spot and vertical streak at the left record the duration of the exposure (0.07 second). The siphon retracts nearly 2 seconds after the exposure. The slight irregularity in the siphon record immediately following the exposure is due to an accidental vibration of the delicately balanced mirror caused by the movement of the shutter. The tuning-fork marks 0.02 second.

A contraction of the siphon will be shown by an upward movement of the reflected beam. Passing through a shutter is another beam of light. This beam plays on the expanded siphon, and beyond that, directly on the camera slit. On opening the shutter, the beam will

¹ Dr. D. J. Edwards was kind enough to let me use his optical recording apparatus, and to give me much of his time and help in the making of records, for all of which I wish to express my gratitude.

therefore stimulate the animal, and at the same time record the exact duration of the exposure on the moving film. The shadow of a vibrating tuning-fork is also focused on the slit, so as to furnish a time record.

One of the records obtained in this manner is given in Fig. 2. With an exposure of 0.07 second at this intensity, the retraction of the siphon appears only after a latent period of nearly 2 seconds. A record like that of Fig. 2 is striking testimony of the composition of the reaction time. Since it is our purpose to study the photochemical aspect of photic sensitivity, our attention must therefore be devoted to the relatively short exposure period during which the reception of the light takes place.

III.

Before taking up the nature or the details of the experiments, it is necessary to describe the apparatus which is used for the accurate control of short exposures. In principle the mechanism is that of a focal plane shutter. Its construction may be described with the help of the three views in Fig. 3. Essentially the shutter consists of a piece of black cardboard C, having a variable aperture A which moves with a definite speed past an opening O, through which a beam of light is directed. The duration of the exposure depends on the velocity of the cardboard and the size of the aperture. The rest of the apparatus serves merely to control these two factors accurately and easily.²

The variable aperture is obtained by using slides S, each having a different sized opening—all slides, however, having the same weight. A slide is placed in the apparatus by slipping it into the raised grooves G; it is kept in place by them with the help of the raised end-piece P. The front of the shutter may be easily removed for the exchange of slides by turning the thumb screws T. The cardboard C which holds the slide is attached at the bottom to a cylindrical wooden rod R. As it moves across the field the cardboard slides in a simple groove at the top of the shutter, and the wooden rod slides in a metal tube Z

² I made this apparatus by converting a focal plane shutter originally constructed by Mr. J. G. Hubbard. I take this opportunity of thanking Mr. Hubbard for much in the way of advice and material assistance in the building of apparatus used in this work.

at the bottom. When it is at the left of the shutter, the rod is held by a small projecting spring clip F, which may be released by hand, or more conveniently by an air bulb. To the ends of the cylindrical

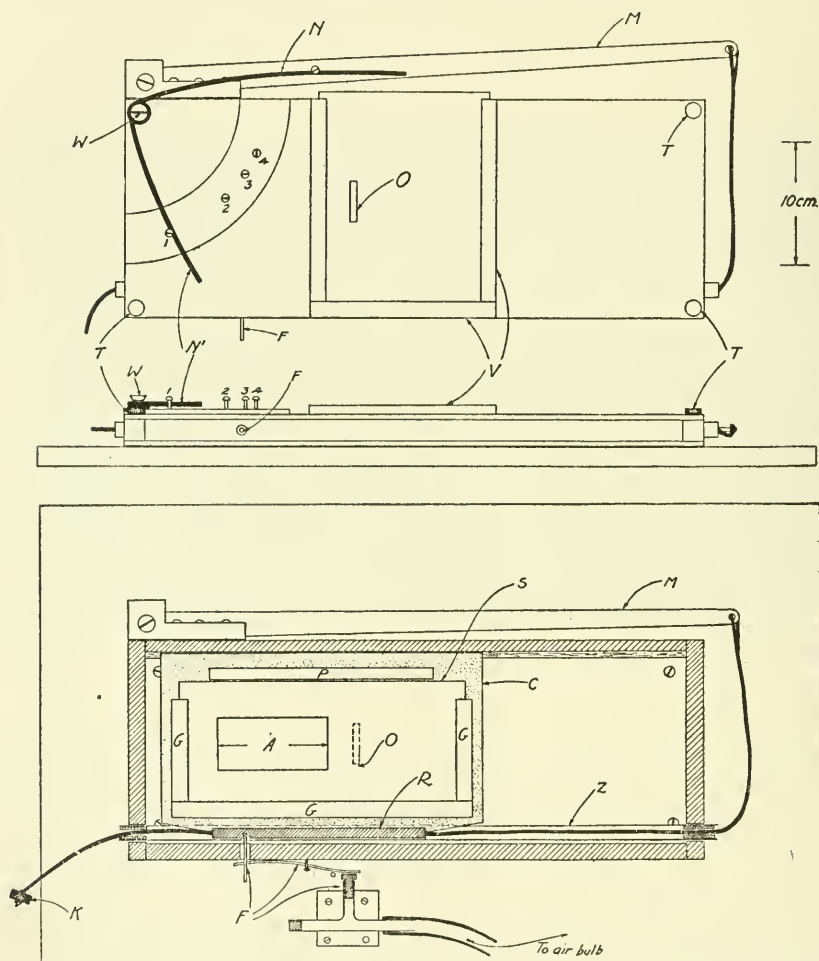


FIG. 3. Three views of the shutter. The upper drawing is the front view; below that is the bottom view; and below that is a partial section through the shutter. The letters are explained in the text. In the upper drawing the details of the spring release clip F are omitted, because they are given in the lowermost view. Similarly the board to which the shutter is attached is also omitted from the upper drawing.

rod R is attached strong twine, which on the left connects with a knob K, and to the right leads to the projecting bar M. Therefore an upward movement of the bar results in the sliding of the cardboard across the length of the shutter.

The motion of the projecting bar is due to the release of the heavy brass wire spring NN' wound several times around the screw W. The tension of the spring and therefore the speed of the shutter may be varied by placing the arm N' of the spring against the appropriate raised screw head 1, 2, 3, or 4. The calibration of this movement is accomplished by recording the excursion of the tip of the bar M on a rapidly moving kymograph. Several such records gave identical results. A few hundredths of a second after being released, the shutter slide moves with a constant velocity, which in these experiments is 67.5 cm. per second. The exposure may therefore be computed from the width of the aperture in the slide, by multiplying the width by the fraction of a second during which the slide moves 1 cm. (= 0.0148 second). The front and rear boards of the shutter have raised grooves V for the insertion of cards having different sized stationary openings O. In this manner, the dimensions of the beam of light may be varied. In these experiments the size of the stationary opening is 5×35 mm.

To get the shutter ready for use, the opening O is temporarily covered with a black card, and the knob K is pulled until the shutter slide C is brought to the left of the shutter. Here it is caught by the release clip F and held in position. The movement of the shutter slide C of course pulls the bar M down to the top of the shutter against the tension of the spring NN'. The temporary card is then removed, and the shutter is ready for an exposure. At the proper moment the spring clip is released, and at once the spring NN' exerts its action. The bar M is jerked rapidly upward and the shutter slide C is shot past the opening O, thus effecting the proper exposure.

When set up, the shutter as a whole is placed in the front wall of a light-tight compartment Y (Fig. 4), containing a 250 watt concentrated-filament Mazda lamp. The center of the beam through the opening O is a few millimeters above the level of a long table B. This is so that the beam will be exactly centered on the siphon of an animal in a dish of sea water standing on the table. The general arrangement

of the apparatus is shown in Fig. 4. The top of the table B is painted black except for a white streak parallel to the long axis of the light beam. The white streak is a background on which to view the movement of the siphon. In addition the streak is graduated in centimeters marking distances from the center of the source of illumination. In this way the siphon of the animal may be placed at exactly the desired distance from the light, where it will receive the proper exposure from the shutter.

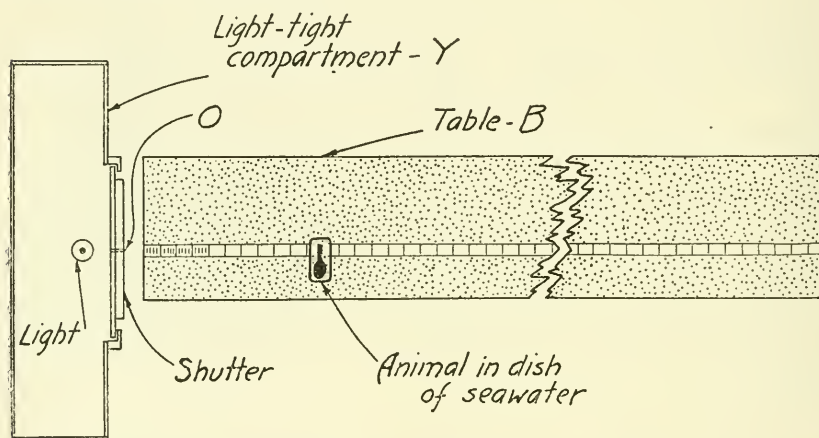


FIG. 4. Arrangement of apparatus in the dark room. The air bulb attached to the spring release clip of the shutter by means of rubber and glass tubings is not shown in the figure. With the help of this bulb and tubing, an exposure can be effected from any position at the work table.

IV.

The first experiments with which I undertook to test the photochemical nature of the light sensitivity were concerned with the relation between the exposure time and the minimum intensity necessary to produce a response. Since the discovery of the Reciprocity Law by Bunsen and Roscoe in 1862, its validity has been demonstrated for a variety of purely photochemical reactions. It obtains whenever the quantity of photic energy necessary to produce a given chemical effect is constant, whether the intensity is low and the exposure long, or the reverse. There are complex reactions, partly photochemical

in nature, which do not obey the reciprocity rule (Schwarzschild, 1899). However, when a process does proceed according to this dictum, it is highly probable that its basis is a simple photochemical reaction.

The usual procedure in investigations of this kind is to vary the intensity and to determine the time required to produce a given effect. This course was adopted with *Ciona*, and the results bore out the Bunsen-Roscoe expectation (Hecht, 1918-19, *a*). Such a technique is entirely out of the question with *Mya*. The exposure required is so short that the errors of measurement would be too great. It is simpler to keep the exposure time under control, and measure the minimum intensity necessary to elicit a response with different exposures. Six exposures were chosen for experimentation. Their durations are given in Table I, first column.

TABLE I.

Relation between Exposure Duration and Minimum Intensity Necessary to Elicit Response.

Exposure (<i>t</i>).	Intensity (<i>I</i>).	<i>I-t</i>
<i>sec.</i>	<i>meter candles</i>	<i>meter candle sec.</i>
0.016	334	5.34
0.023	238	5.47
0.030	194	5.82
0.053	112	5.94
0.073	76	5.55
0.104	54	5.62
Average		5.62

Eight animals which had been thoroughly dark-adapted were used. Taking a given exposure, I subjected each animal in turn to it, and noted whether it responded or not. After each animal had been given a rest of at least 15 minutes, it was again exposed, nearer or farther from the light depending on the individual's previous response. In this way the distance at which a response was elicited was gradually approximated to the distance at which no response could be elicited. I considered a determination as finished when the "no response" distance was 1 cm. farther than the "response" distance. Frequently

the last readings were repeated a few times. An example of an experiment which will indicate the procedure is given in Table II. The entire series of experiments took 6 days, the same animals being used throughout. They were in good condition after the experiments, and remained alive in the laboratory for many days afterwards.

From the determination of the individual minimum distance, it is simple to calculate the minimum intensity by the inverse square law. This was done for each animal for every exposure before an average

TABLE II.

Determination of Minimum Stimulating Distance.

Animal 120, July 18, 1919, exposure, 0.053 second.

Time.	Distance.	Response.
	<i>cm.</i>	
10.07	90	R.
10.34	100	R.
10.52	100	R.
11.14	120	R.
11.41	140	R.
11.57	150	R.
12.18	150	R.
12.34	160	N. R.
2.22	155	N. R.
2.46	152	R.
3.00	153	R.
3.16	154	N. R.

R. = Response elicited.

N. R. = No response elicited.

value was made. A summary of the experiments, giving the average figures, is shown in Table I and graphically in Fig. 5. The third column of Table I shows clearly enough that the Reciprocity Law of Bunsen and Roscoe holds true for this form of photic sensitivity. The curve drawn in Fig. 5 is a theoretical one on the assumption that the photochemical effect (E) is a function of the intensity (I) and the exposure (t), so that

$$Ek = I \cdot t = 5.62$$

k being a constant. The deviations of the average experimental points from the theoretical hyperbola of Fig. 5 are not great. In fact the probable error of any *individual* determination is 6.7 per cent of the mean value assumed in drawing the hyperbola in Fig. 5.

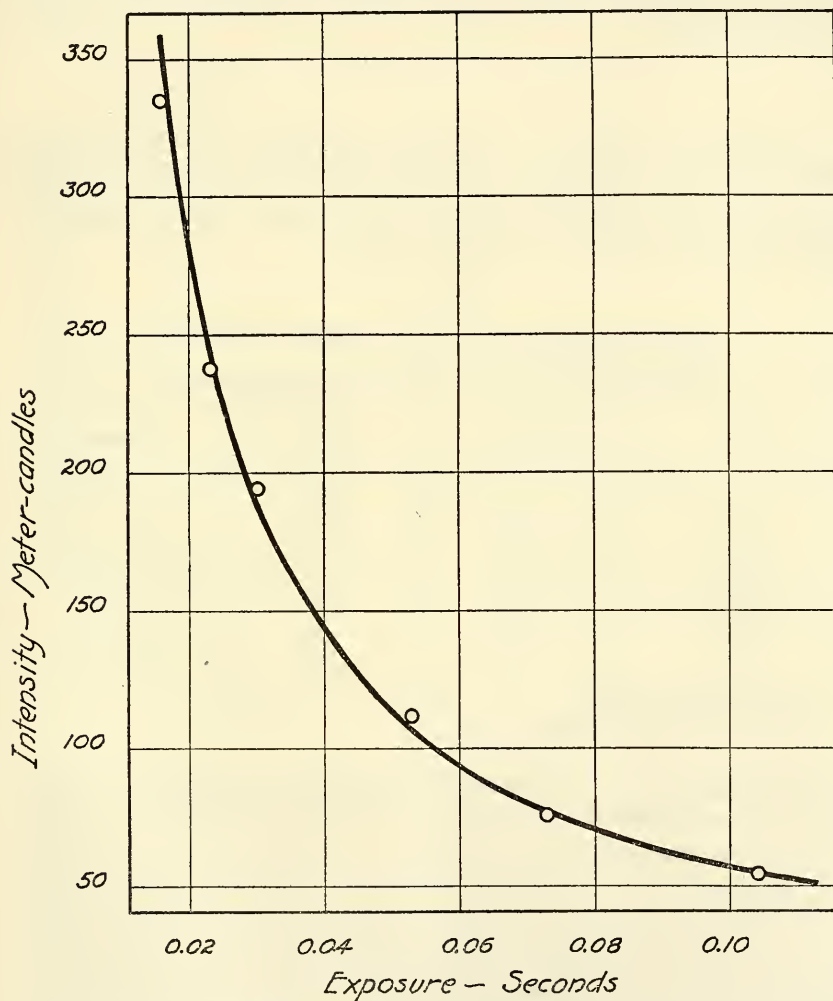


FIG. 5. Relation between exposure and minimum intensity necessary for a response. The points are the average experimental values. The curve is an hyperbola, $I \cdot t = 5.62$, drawn from the theoretical expectation of the Bunsen-Roscoe law.

On the basis of these results, there seems good reason to believe that the initial action of the light on the sense organ is photochemical in nature. This is precisely what we have assumed in our hypothesis. However, there is still another test possible in this connection, and it was also applied in the study of the effect of the light on the photo-sensory process.

V.

It is almost axiomatic to say that photochemical reactions possess low temperature coefficients, very near 1.00 for 10°C. (Sheppard, 1914, p. 304). The obvious thing, therefore, is to determine the effect of the temperature on photoreception.

TABLE III.

Minimum Stimulating Distance at Different Temperatures.

Animal 186, August 20, 1919, exposure, 0.016 second.

Time.	Temperature.	Distance.	Response.
	°C.	cm.	
11.05	24.3	57	R.
11.10	24.5	58	N.R.
11.15	24.3	57	R.
11.20	24.9	58	N.R.
11.25	24.5	57	R.
11.30	24.1	58	N.R.

R. = Response elicited.

N. R. = No response elicited.

Here again it is of the utmost importance to distinguish between the two periods of the reaction time. The reaction time as a whole possesses a high temperature coefficient. For this, however, the effect of temperature on the latent period is entirely responsible (Hecht, 1918-19, *d*). Therefore, if our results are to be of the desired significance, we must study the relation between the temperature and the occurrences in the exposure period only. For the same reasons as before, this resolves itself into a determination of the minimum intensity necessary to elicit a response at different temperatures with a given exposure.

The experiments were conducted as previously outlined, except that 5 minutes were allowed between tests. This time has been found sufficient for the complete recovery from a single stimulus. Using a constant exposure of 0.016 second, I determined the minimum intensity necessary to elicit a response at four different temperatures. When the minimum stimulating distance was found, the determina-

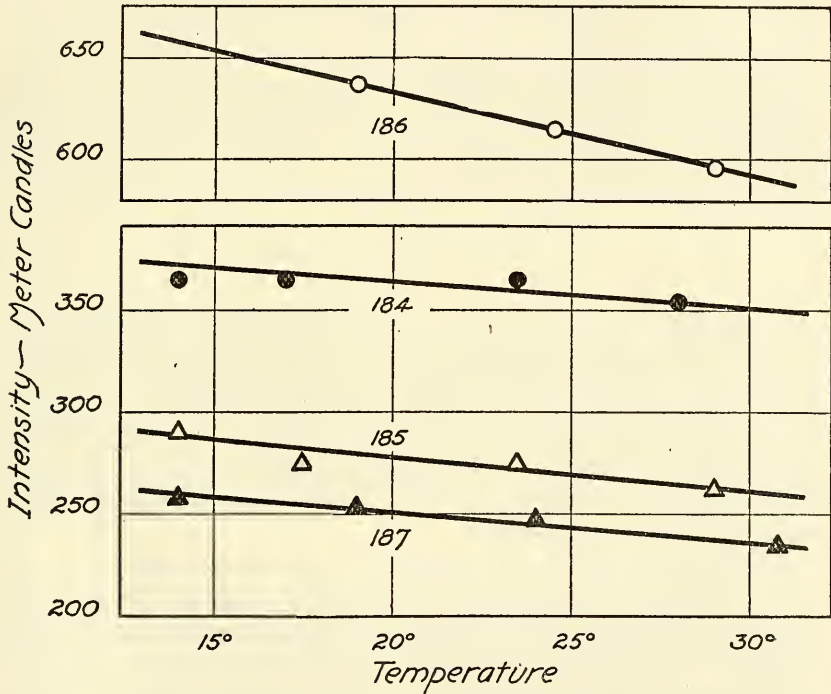


FIG. 6. Relation between the temperature and the minimum intensity necessary for stimulation. The points are single determinations for each experiment shown.

tion was checked at least twice. Table III gives the details of a portion of one such experiment, wholly typical of the others. Proper precautions were, of course, observed for the maintenance of a fairly constant temperature, etc. In this respect *Mya* is a particularly good experimental animal, because it helps to stir the sea water by means of its own continuous water current.

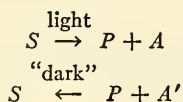
After some preliminary tests, four carefully controlled experiments were made, several days apart. The results were so uniform that further experimentation was deemed unnecessary. The data for the individual experiments are given graphically in Fig. 6. It will be seen that the effect of the temperature of the animal on the minimum stimulating intensity is practically negligible. The temperature coefficients for 10°C. (15–25°), calculated in the ordinary way from Fig. 6, are 1.04, 1.06, 1.07, and 1.06 respectively for Experiments 184, 185, 186, and 187. These values are so characteristic of endo-energetic photochemical reactions, that, combined with the applicability of the Bunsen-Roscoe law, they can lead to but one conclusion. This is that the initial effect of the light in photic stimulation is a purely photochemical phenomenon rather simple in nature.

VI.

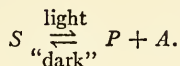
As a result of these two sets of experiments we are justified in accepting the proposed hypothesis with a reasonable degree of confidence. Of course the hypothesis is not final. At each step in its construction I have usually suggested an alternative which is less simple than the one eventually adopted. Therefore, it may be necessary to make alterations in the details of the hypothetical chemical system as further evidence accumulates.

One consideration, however, remains of paramount significance and must be the basis of any possible explanation of this kind of photosensitivity. This is that the mechanism of photoreception is not a single process. Corresponding with the division of the reaction time into an exposure or sensitization period and a latent period, there is a fundamental division of the underlying machinery into an initial photochemical reaction and a consequent ordinary chemical reaction. This duality is patent in every experiment with *Ciona* and *Mya*.

Whether the initial photochemical reaction is strictly reversible or only pseudoreversible depends in a large measure on the relation between the primary and secondary reactions of photoreception. It is often as compatible with the data to assume the primary reaction to be



in which A' is an accessory precursor different from A , as it is to assume it to be



If, as we have postulated, the secondary is catalyzed by the products of photolysis of the primary reaction, then the primary reaction is to be considered as strictly reversible. If, however, the reaction between the two is conceived to be in the nature of a catenary chemical interaction, then a pseudoreversible reaction is a more likely possibility for the primary process than a strictly reversible one. Even in this instance, a photochemical reaction is feasible, if the secondary process itself is assumed to be reversible.

None of these alternatives, however, appear very attractive because of their innate complexity. This becomes especially true when it is necessary to make a mathematical analysis of data in order to compare theoretical expectation and actual performance. The proposed hypothesis of photoreception is therefore to be preferred in its present form, unless some glaring discrepancy arises.

VII.

Aside from their significance with regard to an hypothesis of photoreception, these experiments involve some general conceptions of rather wide application. In the study of the responses of organisms much agitation has resulted over the difference between those individuals which are sensitive to the continuous action of light, and those which are sensitive to a rapid change in the intensity of the light only. The distinction as it is usually made rests on the assumption that for the one group the effective stimulus is a definite quantity of light, whereas for the other group the effective stimulus is the *rate of change* of the intensity. The conception underlying this differentiation, however, is as fallacious as it has been common. I have no desire to enter into a controversy which has been continued much too long. But the

experiments on *Ciona* (Hecht, 1918-19, *a*) and *Mya* have demonstrated that the distinction on which this controversy rests is without any but the most superficial basis.

The facts are simple enough. Blaauw (1909) and Fröschel (1909) demonstrated that the orientation of certain plants obeys the Reciprocity Law of Bunsen and Roscoe; therefore a definite amount of light is required for a stimulus. Loeb (1918) and his associates proved the same to be true for the animals with which they experimented. All these organisms are, of course, to be classed in the group which responds to an obviously continuous source of illumination. On the other hand, *Mya* and *Ciona* belong decidedly in the group of organisms which is sensitive to light only when the illumination intensity has been increased rapidly. And yet both *Mya* and *Ciona* must receive a definite amount of light before they respond. In *Ciona* this is approximately 5,000 meter candle seconds; and in *Mya*, as we found in this paper, the required energy is about 5 meter candle seconds. The stimulus for both groups of organisms is therefore the same, a definite quantity of light energy producing a specific photochemical effect. Certainly no distinction can be made between them on this score.

One point remains to be elucidated. Since both groups of organisms require fundamentally the same stimulus, how is it that animals like *Mya* and *Ciona* respond apparently to sudden illumination only? This is indeed a paradoxical situation, yet the answer to the question is simple. The explanation depends on the reversible character of the sensory process.

The presence of a regenerative mechanism in the sense organ is shown by the course of dark adaptation. The photosensitive material decomposed by the light is automatically regenerated as soon as some products of decomposition accumulate. The velocity of this regenerating reaction depends on the concentration of the precursor decomposition products. Therefore a little time must elapse before the effect of the regenerating reaction will become apparent. It is precisely during this short interval of time that the required amount of energy must be received by the sense organs in order to produce a response.

Physically speaking, this energy requirement means that a definite mass of sensitive substance is decomposed by the light. When the energy is delivered rapidly, that is when a high illumination is attained suddenly, the necessary photosensitive substance is decomposed before the regeneration reaction sets in. But if the energy is delivered slowly, sufficient sensitive substance to produce a response cannot be decomposed before the "dark," regeneration reaction becomes effective. The "dark" reaction, once under way, proceeds slowly or rapidly depending on the rate at which the light decomposes the sensitive substance into its precursors. As a result, a stationary state is attained analogous to a condition of true equilibrium, in which no amount of exposure to light can form enough precursors to initiate a response. Even if the intensity continues to increase to a very high value, no effect can be produced as long as the increase is slow, simply because a new stationary state of the opposing reactions will be reached if enough time is allowed.

The time interval during which the necessary amount of energy must be delivered is known experimentally. In *Ciona* the 5,000 units of energy must be received within about 10 seconds; in *Mya* the required five units must enter the sense organ in less than 1 second. The time limit is thus greater in *Ciona* than in *Mya*. This time interval, as we have said, depends on the speed with which the regeneration reaction proceeds. Therefore the regeneration reaction in *Ciona* should be slower than in *Mya*. This is indeed the situation. Dark adaptation is wholly dependent on the regeneration reaction (Hecht, 1918-19, *a*, *b*). In *Ciona*, dark adaptation requires about 4 hours, whereas in *Mya* the same process is complete in about 35 minutes. The time limit for the energy delivery is thus proportional to the velocity of the "dark" reaction which regenerates the sensitive substance.

The conclusion is clear. In spite of the apparent sensitivity of animals like *Mya* and *Ciona* to sudden illumination the effective agent for this sensitivity is a definite quantity of energy which obeys the Reciprocity Law of photochemistry. The fact that this amount of light energy must be received by the sense organs in a limited time is merely a concomitant of the additional circumstance that the photochemical reaction is reversible. The *rate of change* of the light inten-

sity is decidedly not the effective stimulus. Therefore the controversial division of organisms into the two groups previously mentioned, though traditionally sacred, is fundamentally without significance. The effective stimulus in photic irritability is of the same nature whether the response is to a light which is obviously continuous, or to a light whose intensity is suddenly augmented.

SUMMARY.

1. In order to produce a response in *Mya*, the minimum amount of light energy required is 5.62 meter candle seconds. This energy follows the Bunsen-Roscoe law for the relation between intensity and time of exposure.

2. The necessary minimum amount of energy varies but little with the temperature; the temperature coefficient for 10°C. is 1.06.

3. In view of these facts it is concluded that the initial action of the light is photochemical in nature. This substantiates the hypothesis previously suggested to account for the mechanism of photoreception.

4. The constant energy requirement for stimulation of *Mya* shows that the traditional division of animals into those which respond to a constant source of light and those which respond to a rapidly augmented light is without any fundamental significance for sensory physiology.

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THE FREE ENERGY OF BIOLOGICAL PROCESSES.*

PRELIMINARY PAPER.

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(Received for publication, October 5, 1919.)

Any process in nature is accompanied by a transfer of energy. Part of this energy can be used for doing useful work; part of it is dissipated or rendered latent. Moreover, in the burning of a substance, such as coal, only a small percentage of the available energy is at present utilized; the bulk of it is lost in the form of heat. This immense loss of energy is no doubt due to the clumsiness of our technique and to the imperfection of the work engines now in common use. This leads us to inquire whether similar processes in living organisms are more efficient. An investigation has therefore been undertaken to find the ratio of the energy supplied and the maximum work obtainable in life processes. As a suitable subject for this purpose, it was decided to study the fixation of nitrogen through the agency of microorganisms, such as *Azotobacter*.

Every biological process presents three problems for solution: first, the transfer of energy, which depends solely on the initial and final states of the system; second, the mechanism of the reactions involved; and, third, the rates of these reactions.

Up to the present time, biologists and agriculturists have been interested chiefly in the mechanisms and the rates of life processes. However, from this it must not be inferred that they have underestimated the importance of the study of energy relations in life processes. Such a study requires a thorough knowledge of general chemistry (organic and inorganic), and of thermodynamics and the application of its principles to chemical processes. In this day of

* This investigation is being conducted under the direction of Dr. Charles B. Lipman and with the cooperation of Dr. A. R. Davis of this laboratory.

specialization, it would be too much to expect biologists to possess this knowledge in addition to the mastery of their own special field. They are, however, willing to cooperate with physical chemists to bring about the desired progress in biological science. Unfortunately, whenever they present their problems to the physical chemist, they are always met with the statement: "Biological processes are so highly complex that they are not susceptible to thermodynamic treatment."

Biologists and agriculturists cannot, of course, get satisfactory information on this subject from text-books on physical chemistry, since these embody principally the views and guesses of physical chemists of past generations and are interesting from an historical standpoint only. In fact, it was only within recent years that the first serious attempt was made to base even chemical calculations systematically on thermodynamic principles. This was done by Lewis,¹ and Lewis and Gibson.² It is hoped that by the use of their methods we may be able to do for general physiology, in a measure at least, what Lewis has done for general chemistry.

In order to find the maximum or free energy of any chemical process, such as the transformation of mannite into carbon dioxide and water, which certain bacteria utilize for the work required in decomposing sulfates, phosphates, nitrates, or in the "fixing" of nitrogen, we must know not only the change in the heat content attending the process, but also the change in entropy from the absolute zero up to the given temperature, T , at which the process takes place. We can then calculate the free energy of the process with the aid of the thermodynamic equation

$$\Delta F = \Delta H - T\Delta S \quad (1)$$

where ΔF denotes the free energy charged, ΔH the change in heat content, and ΔS the change in entropy of the isothermal process in question.

The thermochemical data found in the literature, although very extensive, are, with few exceptions, extremely inaccurate, while the measurements of usable entropies have only recently been started

¹ Lewis, G. N., *J. Am. Chem. Soc.*, 1913, xxxv, 1.

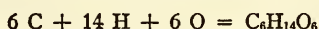
² Lewis, G. N., and Gibson, G. E., *J. Am. Chem. Soc.*, 1917, xxxix, 2554.

under the direction of Lewis.³ However, the compounds in whose entropy Lewis and his coworkers are at present interested are not of the type which suit our purpose. Furthermore, it may take many years before the data which we desire become available. We have, therefore, decided to set up a specific heat apparatus of our own, as well as a high precision bomb calorimeter outfit, for the accurate measurements of heats of combustion of carbohydrates and other organic compounds directly connected with our work. In the meantime, we are conducting experiments with nitrogen fixation bacteria to determine the most favorable conditions for the obtaining of interpretable data.

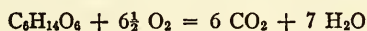
It may be of interest to show just how the free energy calculations of a biological process are made from approximate data found in the literature.

If we wish to find the efficiency of the process of the fixation of nitrogen by *Azotobacter* in a 0.1 molal solution of mannite, it is only necessary to consider the mannite in the thermodynamic calculations, since the concentration of the essential salts added is extremely small compared with that of the mannite itself.

(a) *Calculation of ΔH for the Reaction.*—



This is equivalent to the heats of formation of 6 mols of CO_2 plus 7 mols of H_2O minus that of the reaction



or

$$\Delta H = 6(-94,250) + 7(-68,280) - (-728,400) = -315,060 \text{ calories}$$

(b) *Calculation of $T\Delta S$ for the Same Reaction.*—In order to calculate the entropies of the substances involved in the above reaction, we must know their specific heats from the absolute zero up to $25^\circ\text{C}.$, the temperature at which the reactions take place. The entropy of each substance is related to the average atomic heat at constant volume, C_v , by the thermodynamic equation:

$$S = \int_0^T C_v d \ln T = 2.3 \int_0^T C_v d \log T \quad (2)$$

³ The measurements are being made in the chemical laboratory of the University of California under the direction of Dr. G. N. Lewis.

Although we have sufficient data from which to calculate the entropies of CO_2 and of H_2O , there are no such data available for mannite. We can, however, obtain an approximate value for our present purpose by comparing its average atomic heat at 25°C . (the only value found in the literature) with those of other substances whose entropies are known and whose average atomic heats at 25°C . are approximately of the same magnitude as that of the mannite. For this purpose, we may plot C_p against $\log T$ for solid crystalline C_6H_6 and for graphite. Since for a certain class of substances these curves do not intersect, but approach the absolute zero in the same general way, we may locate between these two curves the average atomic heat curve for mannite.⁴

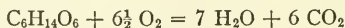
The area under this curve amounts to approximately 0.95. This multiplied by the number of atoms and by 2.3 (see Equation 2) gives 56.8 entropy units. Subtracting from this value the entropies of 14 hydrogen atoms (222.6), 6 oxygen atoms (144.6), and 6 carbon atoms (7.8), we obtain -318 entropy units as the value of ΔS for mannite. We then have for the free energy of formation of 1 mol of solid crystalline mannite from its elements at 25°C .

$$\Delta F = \Delta H - T\Delta S = -315,060 - (-94,760) = -220,300 \text{ calories}$$

Finally to obtain the free energy of 1 mol of mannite when dissolved in 10 kilos of water, we may assume, in the case of substances like mannite, that the activities are proportional to the concentrations, so that for the free energy of dilution from saturation to 0.1 molal

$$\Delta F' = -RT \ln \frac{1.49}{0.1} = -1,600 \text{ calories}$$

Adding this to the above value, we obtain $-221,900$ calories. With this value we can now calculate the available or free energy of the reaction.



or

$$\Delta F'' = 7(-56,620) + 6(-94,360) - (-221,900) = -740,600 \text{ calories}$$

⁴ The average atomic heat for mannite is obtained by multiplying its specific heat, which is given in the literature for 25°C ., by the molecular weight and dividing the product by the number of atoms in the molecule.

This would be the maximum amount of energy at the disposal of the *Azotobacter* in 10,182.1 gm. of solution, 182.1 gm. of which is mannite, if the latter were all converted into CO_2 . To be sure, a certain amount of the carbon of the mannite is utilized by the bacteria in the building of their proteins; the exact amount so combined will need to be determined experimentally. For the present, let us assume that all the carbon in the mannite is converted into CO_2 .

As previously noted in the introduction, a thermodynamical calculation of transfer of energy involves a knowledge of the initial and final states of the system. In the present instance, we have mannite as the initial state—the final state is not yet known. For the purpose of this calculation, we may assume the final product to be hydrated ammonia. Under this assumption, $-740,600$ calories would be sufficient energy to form about 10 mols of ammonia from water and nitrogen gas; that is, if the bacteria acted directly or indirectly merely as catalytic agents. As a matter of fact, it is shown in Greaves⁵ summary of nitrogen fixation by *Azotobacter* that the amount of nitrogen fixed is only about 1 per cent of the weight of mannite, or, in molal proportions, for every mol of mannite used up only about 0.1 mol of ammonia is formed. According to the source above quoted, we would obtain about 0.1 mol of ammonia instead of 10 mols, or only about 1 per cent of the total available energy is utilized in the fixation of nitrogen.

⁵ Greaves, J. E., *Soil Science*, 1918, vi, 181.

APPARATUS FOR MEASUREMENT OF OXIDASE AND CATALASE ACTIVITY.

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(Received for publication, November 25, 1919.)

On account of some undesirable features of the simplified Bunzel oxidase apparatus,¹ the author has designed a simple apparatus (Fig. 1) incorporating the advantages of both the simplified Bunzel apparatus and the original design.²

The simplified Bunzel oxidase apparatus makes no provision for the absorption of CO₂ other than by the reagents used. In some reactions the CO₂ production cannot be disregarded without error. Bunzel gives data on this point.²

This source of error is removed in the new design by using a caustic tube and alkali to absorb the CO₂ produced during the reaction. 1 cc. of 0.1 N alkali is placed in the caustic tube, and on shaking the apparatus the liquid surges back and forth beneath the tube, forcing the air over the alkali.

The apparatus has a volume of 68 cc. measured to the zero of the middle graduated tube. The volume of glass comprising the boat is 3 cc., to which is added 1 cc. of alkali, 2 cc. of plant juice or dilution, and 5 cc. of reagent and buffer solution, totaling 11 cc. In case it is desired to use the apparatus without the alkali tube, the total volume of liquid should be 11 cc. This allows 57 cc. of air space in which a difference of 1 cm. between the mercury levels in the manometer corresponds to a change of 0.75 cc. in volume at 760 mm. pressure. The manometer is graduated for both positive and negative pressures.

The apparatus can be conveniently used for the determination of catalase activity.

¹ Bunzel, H. H., *J. Biol. Chem.*, 1914, xvii, 409.

² Bunzel, H. H., *J. Am. Chem. Soc.* 1912, xxxiv, 303.

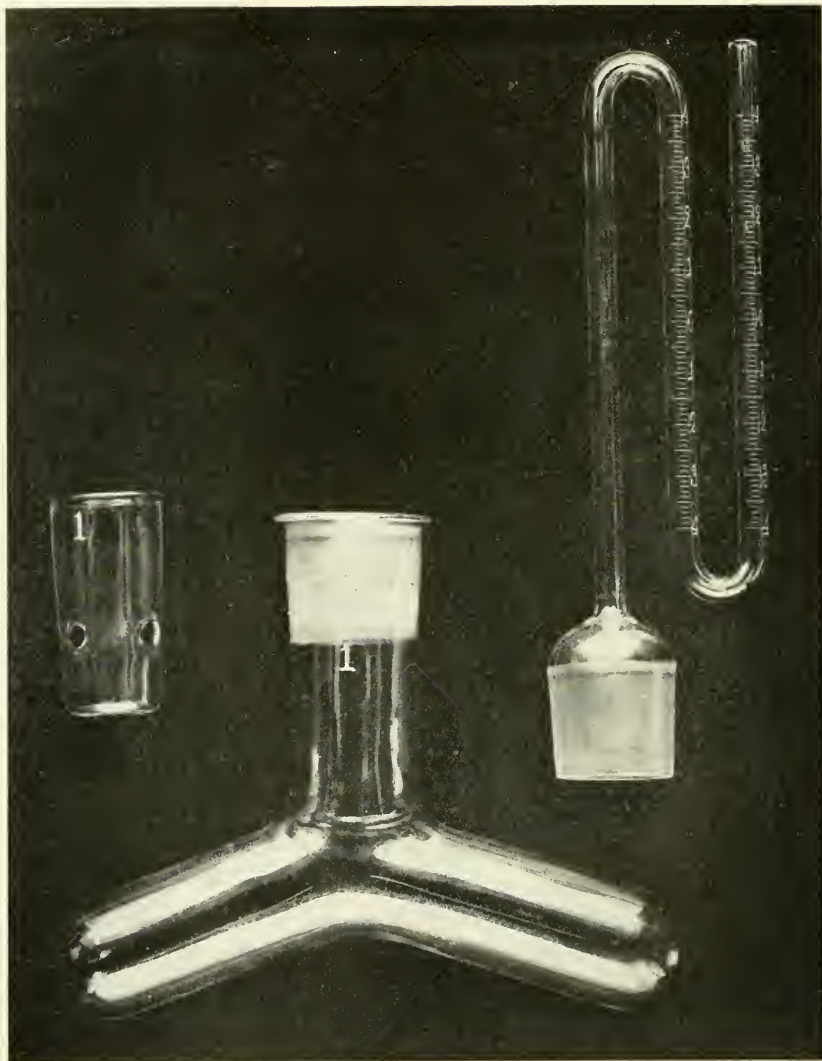


FIG. 1. Simplified apparatus for measurement of oxidase and catalase activity, with tube for CO_2 absorption.

INFLUENCE OF A SLIGHT MODIFICATION OF THE COLLODION MEMBRANE ON THE SIGN OF THE ELECTRIFICATION OF WATER.

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(Received for publication, November 25, 1919.)

I.

When we separate a solution of an electrolyte from pure water by a collodion bag the water will as a rule diffuse into the solution. In a preceding paper¹ it was shown that the forces determining this diffusion are different for concentrations of electrolytes below or above a certain value (which is for certain sodium salts about $M/16$). The forces causing the diffusion of water into the solution below this critical concentration are predominantly electrical, while the forces causing the diffusion above the critical concentration are predominantly (or perhaps exclusively) molecular.

The electrical forces of diffusion depend upon the sign, the valency, a third property of the ions (which we arbitrarily designated as their radius), and in addition upon the concentration of the ions in solution. There is still another variable to be considered; namely, the nature of the membrane. We have already called attention to the fact that a collodion membrane which has once been treated with a gelatin solution shows a different osmotic behavior from a membrane not treated with gelatin. The gelatin treatment consisted in this that the collodion bags with which the experiments were made were filled over night with a 1 per cent gelatin solution (isoelectric or nearly isoelectric). The next day the gelatin solution was poured off and the interior of the bags washed out about six times or more with warm water to remove as much gelatin as possible. They were then put for days into water to dissolve still more and remove the last traces of gelatin.

¹ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 173.

Such bags seemed to retain the effect of the gelatin treatment for a long time, and continued use and subsequent washing did not seem to remove this after effect of the gelatin treatment.

When collodion bags which had received this gelatin treatment were filled with solutions of electrolytes (of the theoretical osmotic pressure of that of a M/64 sugar solution) and were dipped into beakers with distilled water, the influence of the nature of the electrolyte upon the initial rate of diffusion of water into the bag could be expressed by the following two rules.²

1. Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion. The same rule applies to solutions of alkalis.

2. Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion. Solutions of acids obey the same rule.

When the same experiments were repeated with collodion bags which had not come in contact with gelatin, the influence of the electrolytes mentioned in Rule 1 on the diffusion of water was the same as when the membrane had been treated with gelatin. Rule 2, however, was not valid when the collodion membranes had *not* been treated with gelatin. It was of interest to discover the cause of this difference.

The curves in Figs. 1 and 2 show that Rule 1 holds also for collodion membranes *not* treated with gelatin. The solutions of salts used were neutral or slightly alkaline (in the case of Na_3 citrate). The abscissæ in the figures are the logarithms of the concentration, the ordinates the height to which the level of liquid in the manometer rose in the first 20 minutes. The curves in Fig. 1 show that the levels

²Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 717.

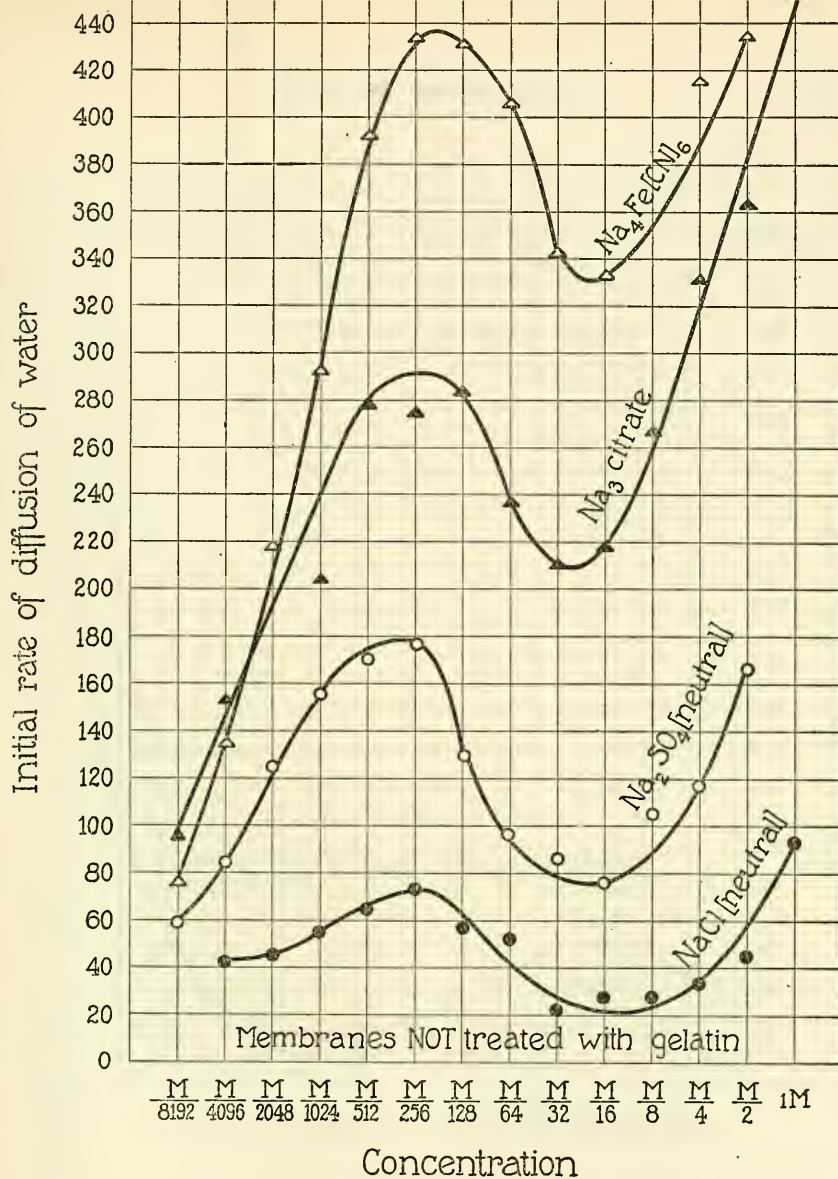


FIG. 1. Curves representing influence of concentration of solutions of different Na salts upon the initial rate of diffusion of water from pure water into solution through membranes not treated with gelatin. The curves are essentially the same as those obtained with collodion membranes which had been treated with gelatin.³ Abscissæ are the logarithms of concentration, ordinates the rise of height of level of solution in 20 minutes. Inside of collodion bag salt solution, outside H₂O.

³ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 177, Fig. 3.

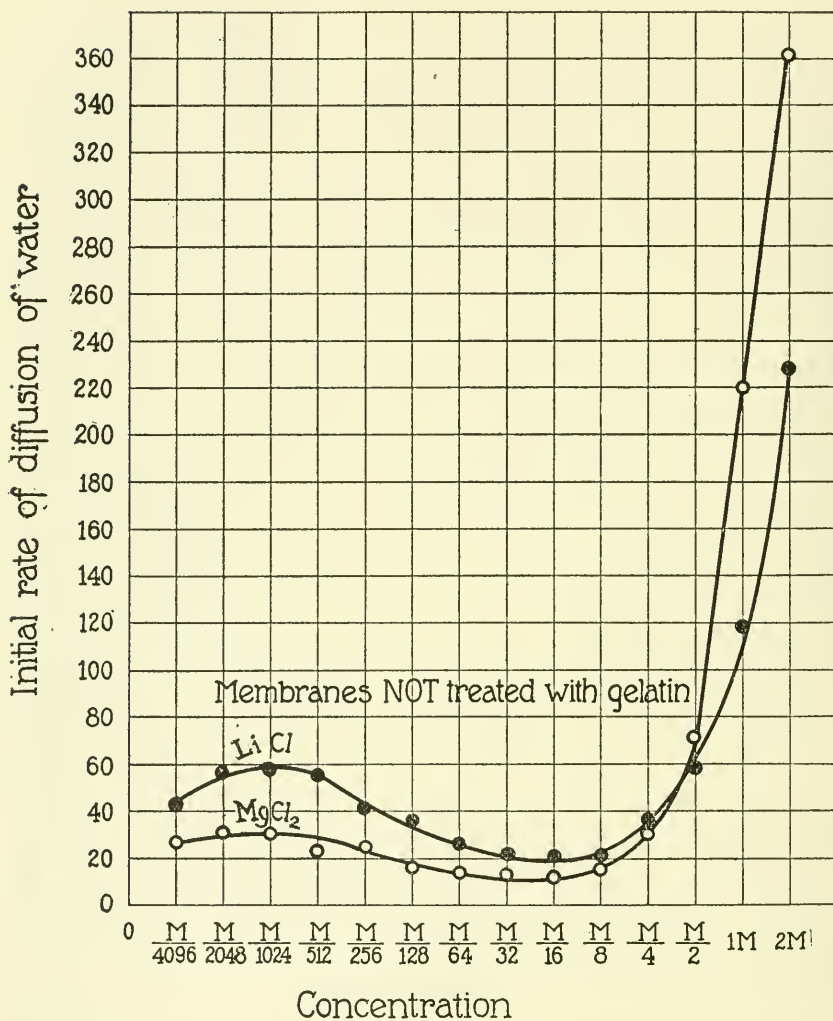


FIG 2. Same curves as in Fig. 1 for LiCl and MgCl₂. Curves identical for membranes treated and not treated with gelatin.

at first rise sharply with an increase in concentration and the more so the higher the valency of the anion. The maximum of the curves is reached at about $M/256$, then the curves fall with a further increase in the concentration until a minimum is reached again at about $M/16$, and then another rise begins. This second rise expresses the gas pressure effect of the solute. When we use membranes treated with gelatin, we get a similar system of curves for the same solutions,¹ and the explanation of the curves is the same for both kinds of membrane. The particles of water diffuse through the membrane as if they were positively charged, being attracted by the anion of the salt and repelled by the cation, the attraction increasing with the valency of the anion. That the repulsion increases with the valency of the cation is shown in Fig. 2 where the lower curve represents the rate of diffusion of water in 20 minutes into $MgCl_2$ solutions and the upper curve the initial rate of diffusion of water in 20 minutes into solutions of $LiCl$ through membranes not treated with gelatin. These curves are also practically identical with those obtained for the same salt solutions when the collodion membrane had previously been treated with gelatin.¹ Hence Rule 1 holds in all essentials equally for membranes treated and not treated with gelatin.

The situation is altogether different for solutions of those electrolytes whose influence is described in Rule 2; namely, acids and neutral or acid solutions of salts with trivalent or tetravalent cation.

When we separate solutions of different concentrations of Al_2Cl_6 from H_2O by collodion bags treated with gelatin, water diffuses very rapidly into the solution and the level of liquid in the manometer rises steeply with an increase in concentration, as is shown by the upper curve in Fig. 3. Water is negatively charged and is powerfully attracted by the trivalent cation Al . When we repeat the same experiment with membranes *not* treated with gelatin (lower curve of Fig. 3), we notice that in that range of concentrations of Al_2Cl_6 where the diffusion is determined chiefly (or exclusively) by electrical forces no rise occurs until the concentration of the solution of Al_2Cl_6 is about $M/64$; at about this concentration the gas pressure effect of solutions of cane sugar begins to be noticeable. It, therefore, looks as if solutions of Al_2Cl_6 showed no electrical but only the gas pressure effect when separated from pure water by a membrane not treated with

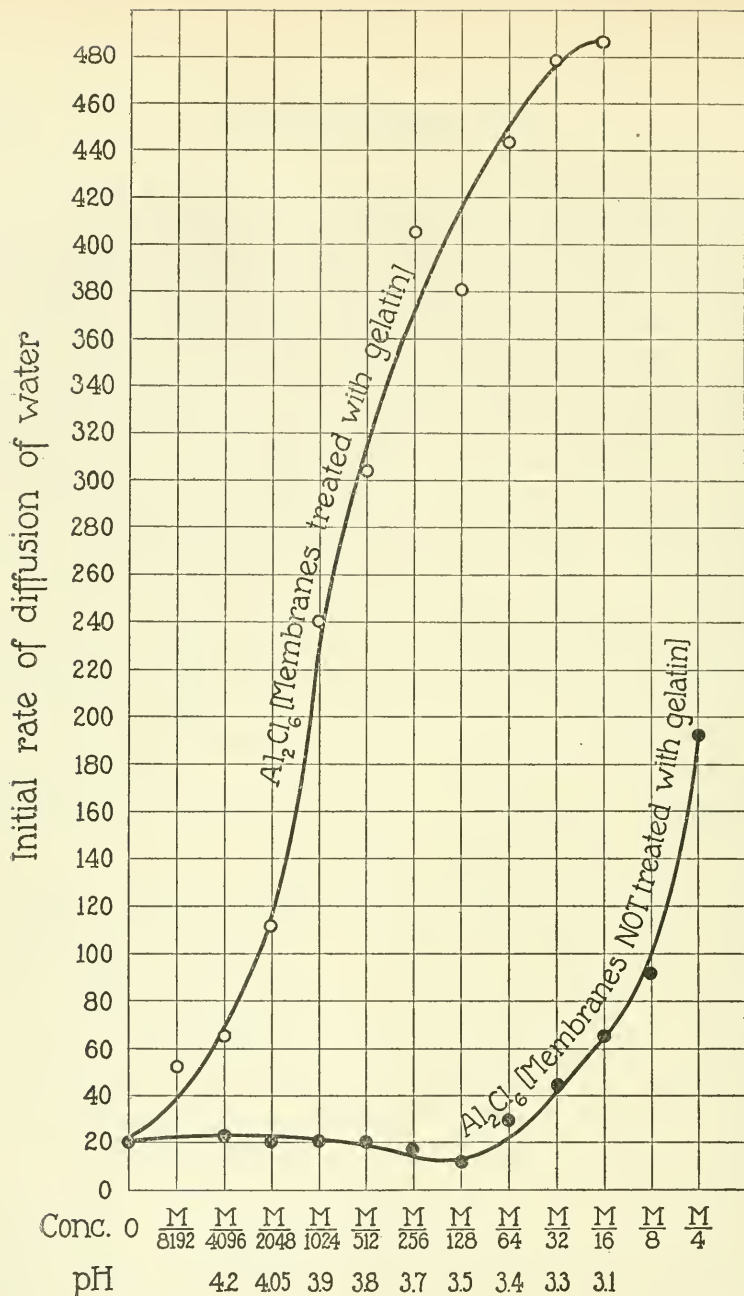


FIG. 3. Showing difference in osmotic behavior of collodion membranes treated and not treated with gelatin. Membranes separating solutions of Al_2Cl_6 from pure water. Upper curve, initial rate of diffusion of water (first 20 minutes) into solution through membranes treated with gelatin; lower curve, diffusion of water during the first 20 minutes into same solution of Al_2Cl_6 through membranes *not* treated with gelatin. In second case only the gas pressure effect of solution is apparent, while electrical attraction of water by solution is lacking.

gelatin. We shall see later that the electrical effect in the lower range of concentrations of Al_2Cl_6 is not lacking, but that the low ordinates are due to the action of the trivalent cation. Curves for the diffusion of water into solutions of LaCl_3 (Fig. 4), which are nearly neutral, show the same difference as those just described for Al_2Cl_6 solutions. When LaCl_3 solutions are separated from water by membranes not treated with gelatin, the ordinates (lower curve in Fig. 4) are low in the region of the electrical effect, while they rise steeply in the same region when the membranes have been treated with gelatin (upper curve in Fig. 4).

The difference in the osmotic behavior of the two kinds of membranes is still more striking when we use solutions of acids. When we separate solutions of strong acids (*e.g.* HCl , HNO_3 , H_2SO_4) from distilled water by collodion membranes previously treated with gelatin, we notice no rise but only a drop (Fig. 5)—negative osmosis—which commences for H_2SO_4 and H_3PO_4 at a concentration of about $\text{M}/256$. In the experiments represented in Fig. 5, the pressure head of the solution of acids inside the bag was about 70 mm. at the beginning. It dropped in 20 minutes to about 10 mm. at concentrations of about $\text{M}/8$ or $\text{M}/4$ for H_2SO_4 and H_3PO_4 (Fig. 5). Owing to the fact that the drop is due to the repelling action of the anion of the acid upon the negatively charged particles of water, the drop in the curves is greater when the anion of the acid is bivalent or trivalent than when it is monovalent. When we separate various concentrations of the same acids from pure water by membranes *not* treated with gelatin we get results of an altogether different order (Fig. 6). Instead of negative osmosis we notice a powerful positive osmosis, *i.e.* a rapid diffusion of water into the solution, and the acids behave almost like the sodium salts with the same anion. By comparing Fig. 1 and Fig. 6 the reader will notice the same steep rise of the curves until the concentration is about $\text{M}/256$; this rise is more considerable in the case of Na_2SO_4 and H_2SO_4 than in the case of NaCl and HCl . In the case of these two salts and acids, the rise is followed by a drop, until for the acids at $\text{M}/32$ and for the salts at $\text{M}/16$ the gas pressure effect of the solution commences. The drop is not noticeable in the case of H_3PO_4 .

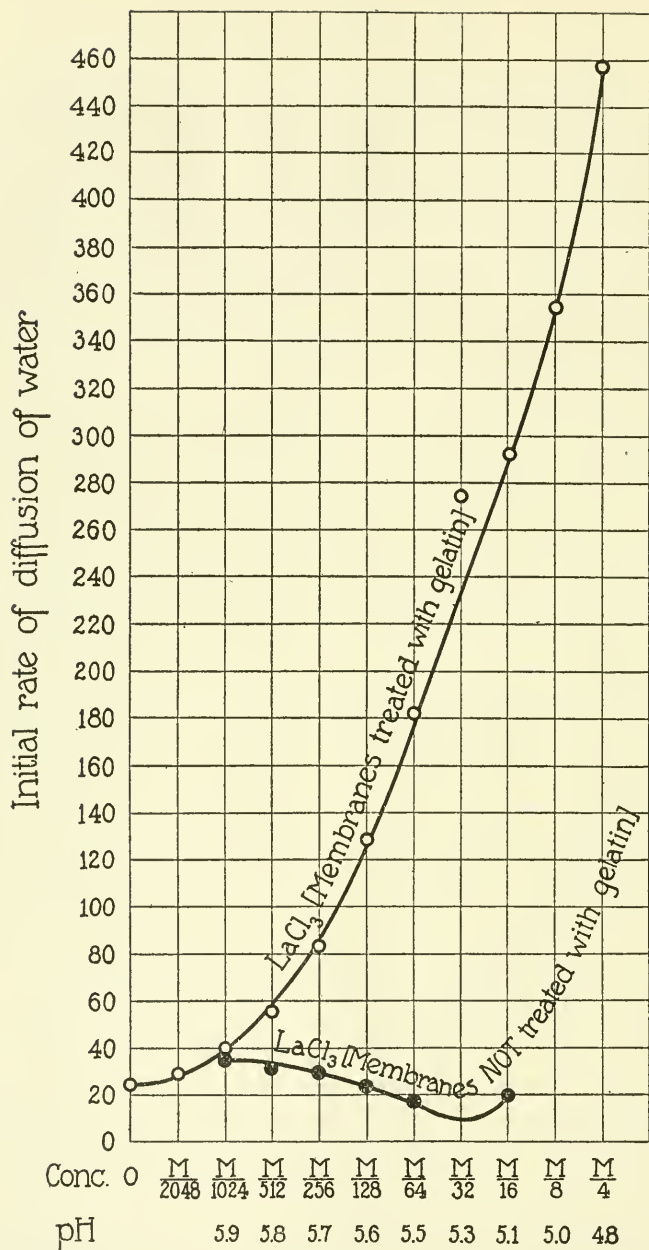


FIG. 4. Showing the same difference of membranes treated and not treated with gelatin for solutions of LaCl_3 .

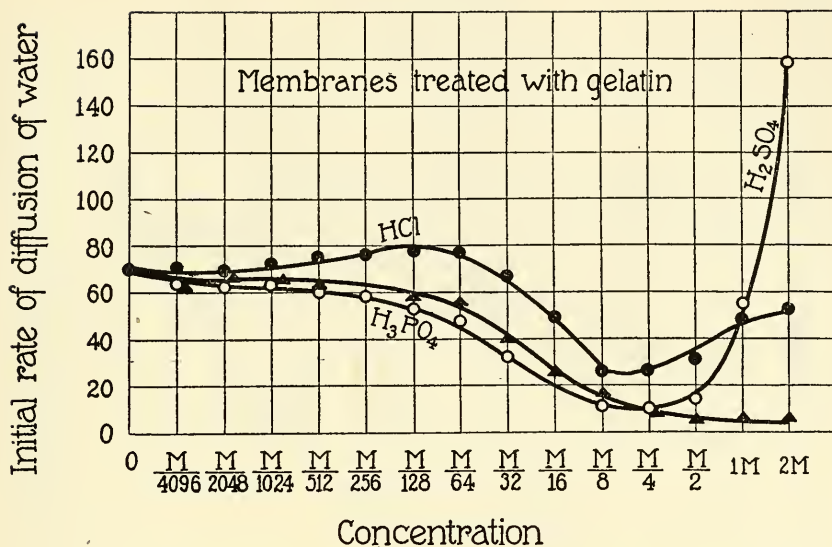


FIG. 5. Negative osmosis when acids (HCl, H₂SO₄, H₃PO₄) are separated from water by membranes treated with gelatin.

Fig. 7 gives the curves for several other acids when the membranes are treated with gelatin and Fig. 8 the curves for the same acids when the membranes are *not* treated with gelatin. When the membranes are *not* treated with gelatin the curve for oxalic acid (Fig. 8) becomes almost like that of Na₂SO₄ in Fig. 1; while the same acid shows the phenomenon of negative osmosis (Fig. 7) when the membrane has received a gelatin treatment previous to the experiment.

II.

What causes this profound difference in the osmotic behavior of collodion membranes according to whether they have or have *not* received a previous treatment with gelatin? It is so customary in biology to explain obscure phenomena by the assumption of variations of permeability that the writer felt it necessary to test the possibility of such an explanation in this case.

The phenomenon of positive osmosis for acid when the collodion bag separating the acid from pure water was not treated with gelatin, and the opposite phenomenon of negative osmosis when the collodion

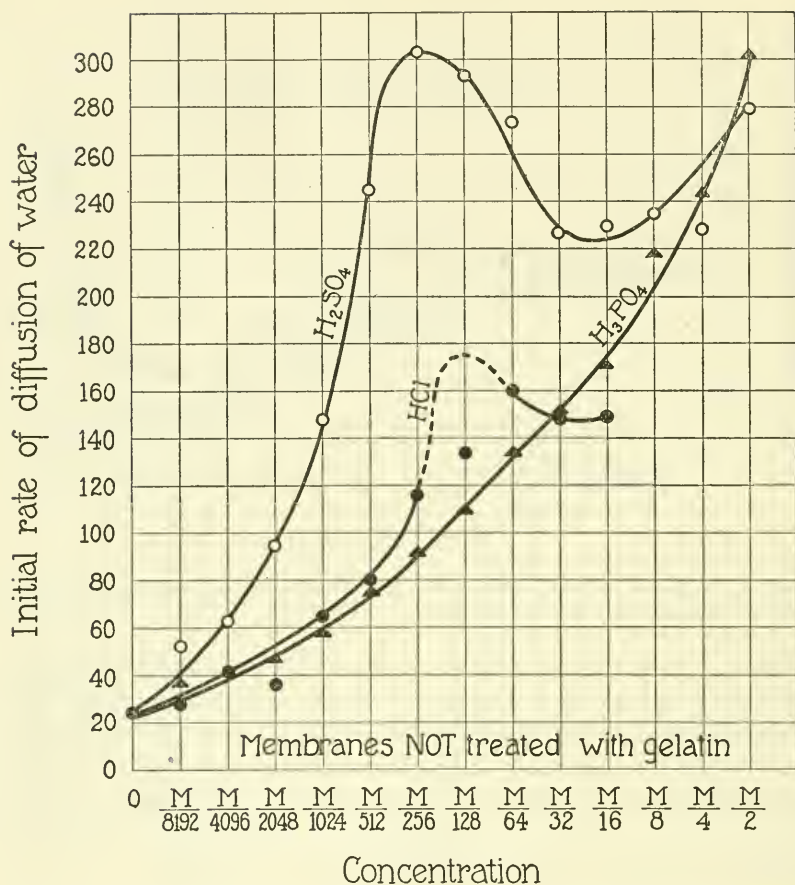


FIG. 6. Positive osmosis when the same acids as in Fig. 5 are separated from water by membranes *not* treated with gelatin. Curves similar to those obtained with sodium salts of the same anion (Fig. 1).

bag separating the acid had been treated with gelatin, suggested that the gelatin treatment had rendered the collodion bag more permeable for acid. It was, therefore, expected that titration of the contents of the bag at the beginning and after 20 minutes would show a greater diminution of the concentration of acid inside the bag when the bag had been treated with gelatin than when it had received no such treatment. The test, however, disproved the correctness of this explanation. *The rate of diffusion of acid from solution into the distilled water was practically the same for collodion membranes treated and not treated with gelatin, as Table I shows.*

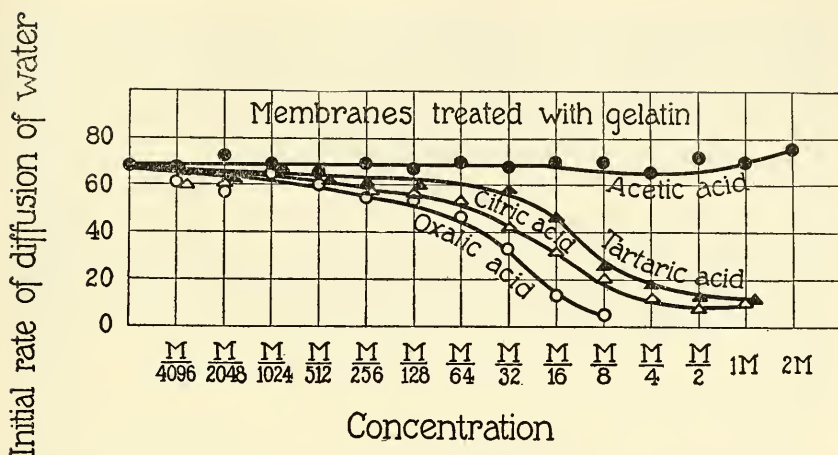


FIG. 7. Negative osmosis when solutions of acids (tartaric, citric, and oxalic) are separated from water by membranes treated with gelatin. The weak acetic acid behaves practically like a non-electrolyte.

The table shows that the amount of acid which diffused out from the collodion bag into the distilled water was (within the limits of accuracy of these experiments) the same for membranes treated and *not* treated with gelatin. Oxalic acid diffused a little more slowly than hydrochloric acid, but the rate of diffusion for each acid is approximately in direct proportion to its concentration. The fact that the same acids caused positive osmosis when the membranes were *not* treated with gelatin and negative osmosis when they were treated cannot be ascribed to differences in the permeability of the two types of collodion membranes.

Since solutions of Al_2Cl_6 and of LaCl_3 caused a vigorous diffusion of water from solvent into solution when the collodion membrane was treated with gelatin, but not when the membrane had *not* received a gelatin treatment, the idea suggested itself that the collodion membranes not treated with gelatin were so permeable for the molecules of the salt that an increase in volume of the solution by a diffusion of water into it was impossible. Titration experiments with Al_2Br_6 proved, however, that there was only a slight difference in the rate

TABLE I.

Loss of cc. of 0.01 N acid for 10 cc. of solution in 20 min.			
	N/32	N/64	N/128
Membranes treated with gelatin.			
HCl.....	10.2	5.0	2.3
H ₂ SO ₄	9.7	4.7	2.2
Oxalic acid.....	7.8	4.0	1.9
Membranes not treated with gelatin.			
HCl.....	11.0	4.9	2.3
H ₂ SO ₄	9.8	4.1	1.7
Oxalic acid.....	8.1	3.4	1.6

of diffusion of Al_2Br_6 through the two types of membranes; the non-treated membrane being slightly more permeable. The difference was, however, much too small to account for the difference in the osmotic behavior of the two types of membranes.

It was, therefore, necessary to look for another explanation in the different osmotic behavior of the two types of membranes. It seemed these differences would find their explanation if it could be proved that water diffuses through collodion membranes not treated with gelatin in the form of positively charged particles even in the presence of acids or salts with trivalent or tetravalent cation. For this purpose the method of electrical endosmose was applied. Identical solutions of LaCl_3 were put inside and outside a collodion bag not treated with gelatin and a current (of about 4.8 milliamperes and about 90 volts) was sent through the solution. The water migrated to the cathode, showing that its particles were positively charged in the presence of LaCl_3 . When the same experiment was repeated with membranes previously treated with gelatin it was found that water is negatively charged in the presence of solutions of salts with trivalent cation. It was furthermore possible to show that in the presence of acids water diffuses through membranes not previously treated with gelatin in the form of positively charged particles.

We can now understand why it is that water cannot diffuse through collodion membranes not treated with gelatin into solutions of salts with trivalent cation, *e.g.* AlCl_3 or LaCl_3 (in the region of electrical effect), since these particles of water carrying a positive charge are

repelled by the trivalent cation. We understand also why the initial rate of diffusion of water through membranes *not* treated with gelatin is greater into a solution of H_2SO_4 than into a solution of HCl , since the positively charged particles of water are attracted by the anion of the acid and the more powerfully the higher the valency of the anion.

A very simple test will demonstrate that the water diffuses through collodion membranes *not* treated with gelatin in the form of positively charged particles even in the presence of $\text{m}/1,000$ acid. We know from Rule 1 (at the beginning of this paper) that positively charged particles

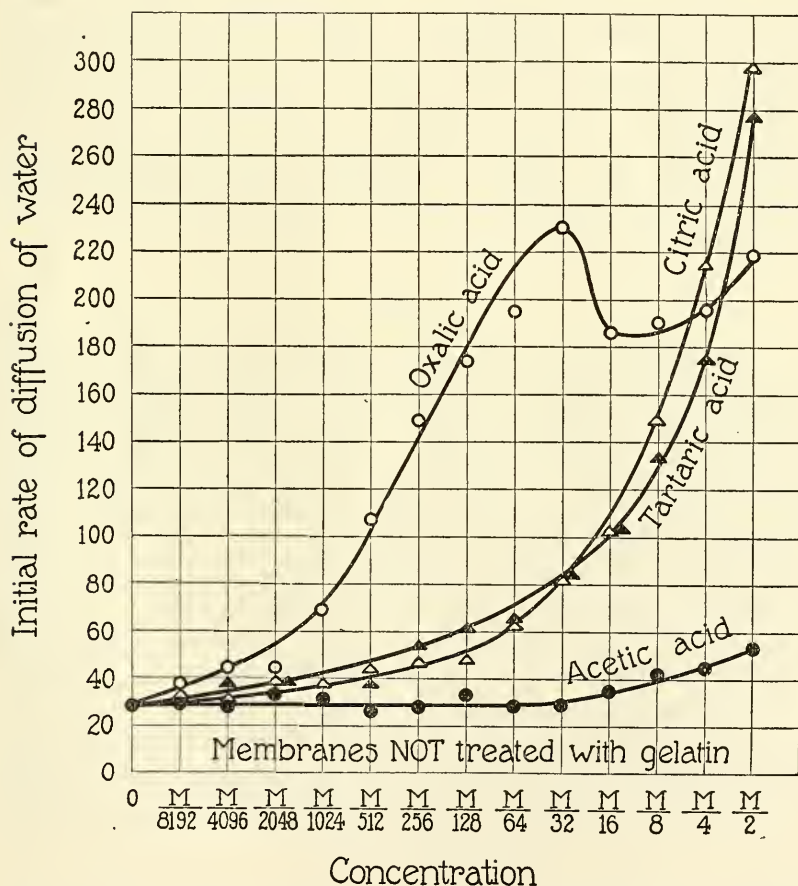


FIG. 8. Positive osmosis when solutions of the same acids as in Fig. 7 are separated from water by membranes *not* treated with gelatin.

of water are attracted more powerfully by Na_2SO_4 , less powerfully by NaCl , and still less powerfully by CaCl_2 . Hence when we make $\text{M}/128$ solutions of these three salts acid through the addition of HNO_3 , and if it is true that water diffuses through the membranes not treated with gelatin in the form of positively charged particles in the presence of strong acid, it should diffuse more rapidly into Na_2SO_4 , less rapidly into NaCl , and least rapidly into CaCl_2 . $\text{M}/128$ solutions of these three salts were brought to a pH of 2.9 through the addition of HNO_3 , and the experiment turned out as the theory demands. When the same experiment was made with collodion membranes treated with gelatin, the order of attraction of the three salts of pH 2.9 for water was reversed, water diffusing very rapidly into the acid solution of $\text{M}/128$ CaCl_2 , less rapidly into the acid solution of $\text{M}/128$ NaCl , and still less rapidly into the acid solution of $\text{M}/128$ Na_2SO_4 , since in this case the particles of water diffusing through the membrane were negatively charged. When the solutions of the three salts were neutral or alkaline, no difference in the osmotic behavior of the two types of membranes was noticed.

III.

In a previous paper¹ the writer had suggested that it was necessary to discriminate between the influence of cations upon the sign of electrification of water and upon the rate with which electrified water diffuses into the solution. This suggestion receives support from this paper, and it is impossible to understand the phenomenon of negative osmosis without this discrimination. Thus it is obvious that both H ions as well as trivalent cations cause the particles of water which normally have a positive charge, to assume a negative charge and it is not yet possible to state whether the H ions or the trivalent cations are more efficient in this direction. It follows, however, from the observations published in this paper that, as far as the rate of diffusion of the charged particles of water into the solution is concerned, the influence of the trivalent cations is much more powerful than that of the hydrogen ions. We express this influence in terms of electrostatic attraction and repulsion between the ions and the charged particles of water, not in order to offer thereby an explanation of this influence but merely to simplify the

presentation of the facts. With this reservation our experiments suggest that the apparent electrostatic effect of the hydrogen ions upon the motion of the electrified particles of water is small if compared with the effect of the trivalent cations; in fact it seems smaller even than that of other monovalent cations.

When acid is put into collodion bags *not* treated with gelatin, we observe a rapid diffusion of water into the acid solution as if the attraction of the positively charged water by the anion of the acid was very strong while its repulsion by the H ion was weak. When we put acids into collodion bags treated with gelatin we observe negative osmosis; *i.e.*, slight attraction of the negatively charged particles of water by the positive hydrogen ion, and a powerful repulsion of the water by the anion of the acid. In all these cases, the apparent electrostatic effect of the hydrogen ion upon the charged particles of water seems smaller than the electrostatic effect of any other ion. When we compare the electrostatic effect of trivalent cations on the rate of diffusion of water, we notice that it is much more powerful than that of the hydrogen ion. When water is negatively charged, it is attracted very powerfully by Al_2Cl_6 as well as by $\text{Al}_2(\text{SO}_4)_3$ though naturally more by the former than by the latter. While in the case of acids the SO_4 ion has a more powerful influence than the H ion, the electrostatic effect of a trivalent cation cannot be overcome by the effect of a bivalent anion. It can only be equalled by the influence of a trivalent anion; *e.g.*, the citrate. This comparatively weak electrostatic effect of the H ion is responsible for the phenomenon of negative osmosis observed in the case of acids. When we use membranes in which the electrified water is negatively charged in the presence of acid the attractive effect of the H ion is so small that the repulsive effect of the anion prevails in the critical range of concentrations, where the drop in the curve occurs. As a consequence no water can diffuse into the solution and the volume of the latter will diminish on account of the diffusion of solute into the pure solvent. Since we notice also negative osmosis in the case of $\text{Ba}(\text{OH})_2$ and $\text{Ca}(\text{OH})_2$, we conclude that the electrostatic effect of the OH ion on the movable stratum of the double layer is also small.

IV.

Gelatin is not the only substance which causes a reversal of the sign of the electrification of the particles of water diffusing through the collodion membrane in the presence of comparatively low concentrations of acid or of salts with trivalent or tetravalent cations. The same effect can be produced when the collodion membrane is treated with 1 per cent solutions of casein, egg albumin, blood albumin, and edestin; while treatment of the collodion membrane with 1 per cent solutions of Fairchild's peptone, of peptone (prepared by Dr. Northrop) from egg albumin digested with pepsin and neutralized, of alanine, of "soluble starch," of "potato starch," and of 0.50 per cent agar-agar did not cause the reversal. The proteins which modify the behavior of the membrane cannot diffuse through the latter but the writer is not yet ready to state that this is the decisive factor. It is of interest that a treatment of the collodion membrane with a 0.002 per cent solution of gelatin makes the membrane already noticeably amphoteric though to a considerably smaller degree than a treatment with a 1 per cent solution of gelatin.

SUMMARY.

1. It is shown that collodion membranes which have received one treatment with a 1 per cent gelatin solution show for a long time (if not permanently) afterwards a different osmotic behavior from collodion membranes not treated with gelatin. This difference shows itself only towards solutions of those electrolytes which have a tendency to induce a negative electrification of the water particles diffusing through the membrane, namely solutions of acids, acid salts, and of salts with trivalent and tetravalent cations; while the osmotic behavior of the two types of membranes towards solutions of salts and alkalis, which induce a positive electrification of the water particles diffusing through the membrane, is the same.

2. When we separate solutions of salts with trivalent cation, *e.g.* LaCl_3 or AlCl_3 , from pure water by a collodion membrane treated with gelatin, water diffuses rapidly into the solution; while no water diffuses into the solution when the collodion membrane has received no gelatin treatment.

3. When we separate solutions of acid from pure water by a membrane previously treated with gelatin, negative osmosis occurs; *i.e.*, practically no water can diffuse into the solution, while the molecules of solution and some water diffuse out. When we separate solutions of acid from pure water by collodion membranes not treated with gelatin, positive osmosis will occur; *i.e.*, water will diffuse rapidly into the solution and the more rapidly the higher the valency of the anion.

4. These differences occur only in that range of concentrations of electrolytes inside of which the forces determining the rate of diffusion of water through the membrane are predominantly electrical; *i.e.*, in concentrations from 0 to about $M/16$. For higher concentrations of the same electrolytes, where the forces determining the rate of diffusion are molecular, the osmotic behavior of the two types of membranes is essentially the same.

5. The differences in the osmotic behavior of the two types of membranes are not due to differences in the permeability of the membranes for solutes since it is shown that acids diffuse with the same rate through both kinds of membranes.

6. It is shown that the differences in the osmotic behavior of the two types of collodion membranes towards solutions of acids and of salts with trivalent cation are due to the fact that in the presence of these electrolytes water diffuses in the form of negatively charged particles through the membranes previously treated with gelatin, and in the form of positively charged particles through collodion membranes *not* treated with gelatin.

7. A treatment of the collodion membranes with casein, egg albumin, blood albumin, or edestin affects the behavior of the membrane towards salts with trivalent or tetravalent cations and towards acids in the same way as does a treatment with gelatin; while a treatment of the membranes with peptone prepared from egg albumin, with alanine, or with starch has no such effect.

INFLUENCE OF THE CONCENTRATION OF ELECTROLYTES ON SOME PHYSICAL PROPERTIES OF COLLOIDS AND OF CRYSTALLOIDS.

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(Received for publication, November 19, 1919.)

I. INTRODUCTION.

When we separate neutral solutions of salts with monovalent cation from pure water by a collodion membrane, water will diffuse into the solution. The writer has shown in a preceding publication¹ that the initial rate of diffusion will at first increase with the concentration of the electrolyte, but as soon as the concentration of the latter is about $M/256$, the initial rate of diffusion of water into the solution will in the case of many electrolytes diminish with a further increase in the concentration of the electrolyte until at a concentration varying between $M/32$ and $M/8$ (according to the nature of the electrolyte) a minimum is reached. This phenomenon is due to the influence of the ions on the electrification and rate of diffusion of water through the collodion membrane. In the presence of neutral solutions of salts with monovalent or bivalent cation, water diffuses through the collodion membrane as if its particles were positively charged and as if they were attracted by the anion and repelled by the cation of the electrolyte with a force increasing with the valency of the ion. With low concentrations of electrolytes the attractive action of the anion upon the positively charged particles of water prevails over the repulsive force of the cation, while, when the concentration exceeds a certain value, which for a number of salts is about $M/256$, the repelling force of the cations of the electrolyte upon the positively charged particles of the water increases more rapidly than the attractive force of the anions. This idea is supported by the fact that the addition of salts with

¹ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 173.

bivalent cation causes a more rapid drop than the addition of a salt with monovalent cation. These phenomena bear so striking a resemblance to the action of the concentration of electrolytes upon the osmotic pressure, the swelling, and other properties of colloids that a discussion of the similarity may seem of interest.

It was found by Pauli² that the addition of a little acid to blood albumin which had been dialyzed for weeks (and which was therefore approximately isoelectric) caused an increase in the viscosity of the protein, which at first was the greater the more acid was added. Very soon, however, a point was reached where the addition of more acid caused again a diminution in the viscosity. The same phenomenon occurs when acid is added to isoelectric gelatin. The addition of a slight amount of acid causes an increase in the osmotic pressure until finally a point is reached where the further addition causes a diminution (Fig. 1). The increase in the osmotic pressure of isoelectric gelatin when a slight quantity of HCl is added, is due to the formation of gelatin chloride, but the depressing effect of the addition of an excess of acid is not so easy to explain. According to Pauli we should ascribe it to the diminution of the degree of electrolytic dissociation of protein chloride due to the increase in the concentration of the common anion Cl. The writer's measurements of conductivity do not support this idea.³ Another suggestion made by colloid chemists is that the addition of more acid causes an aggregate formation of the gelatin particles and therefore a diminution of osmotic pressure. This suggestion rests only on the phenomenon which it is supposed to explain, but it may, nevertheless, be correct. If so, it remains to be explained why an increase in the concentration of electrolytes causes a formation of aggregates.

In a preceding publication⁴ the writer has shown that a 1 per cent solution of gelatin-acid salt, *e.g.* gelatin chloride or gelatin citrate, etc., has its maximal osmotic pressure when the pH is about 3.4 or 3.3. When to gelatin chloride of this pH acid or neutral salt is added, the osmotic pressure (as well as the swelling, viscosity, etc.) falls and the more so the more acid or salt has been added. When we add

² Pauli, W., *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

³ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 483, 559.

⁴ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 559.

alkali, the osmotic pressure falls also, but in this case a complication arises, since the addition of NaOH to a gelatin-acid salt causes a neutralization of the acid and the gradual transformation of the gelatin-acid salt into isoelectric gelatin which has a minimal osmotic pressure.

A 1 per cent solution of metal gelatinate, *e.g.* Na gelatinate, has its maximal osmotic pressure at a pH of about 8.4. When to a 1 per cent solution of metal gelatinate of this pH alkali or neutral salt is added, the osmotic pressure (as well as the swelling and the viscosity) is diminished. When acid is added the same phenomenon occurs but for another reason since the addition of acid lowers the pH and transforms gelatin salt into isoelectric gelatin. We will first discuss the action of electrolytes on the osmotic pressure of metal gelatinate.

II. Water Charged Positively.

Doses of 1 gm. each of commercial, finely powdered gelatin are rendered isoelectric, melted, and made up into 1 per cent solutions of gelatin by adding enough NaOH and water to make the volume 100 cc. The amount of NaOH contained in 100 cc. of each solution varied and it was for different solutions 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of $M/4$ NaOH. Part of this NaOH combined with the gelatin, forming Na gelatinate; the rest remained free. As shown in a preceding paper, there exists a definite chemical equilibrium between the Na gelatinate formed, the isoelectric gelatin, and the hydrogen ion concentration. If the alkali of such a solution is neutralized by an acid (*e.g.* CO_2) or if it is allowed to diffuse out from the solution, the equilibrium is disturbed and some of the metal gelatinate will be transformed into non-ionogenic (isoelectric) gelatin.

These gelatin solutions containing different amounts of NaOH were put into collodion bags. The latter were closed with a rubber stopper which was perforated by a glass tube with a bore of 2 mm. in diameter which served as a manometer to measure the osmotic pressure of the solutions. These bags were put into beakers containing 350 cc. of a solution of NaOH which in each case had the same amount of NaOH in 100 cc. solution as was originally added to the gelatin solution which it surrounded. Thus the 1 per cent solution of Na

gelatinate which had 6.4 cc. of M/4 NaOH in 100 cc. was dipped into a beaker with water which had 6.4 cc. of M/4 NaOH per 100 cc. of H_2O . Since a small part of the NaOH inside the collodion bag had combined with the gelatin, the concentration of NaOH in the outside solution was at first slightly greater than that inside, and as a consequence some NaOH diffused from the outside into the bags. Part of the NaOH in the outside solution was gradually neutralized by

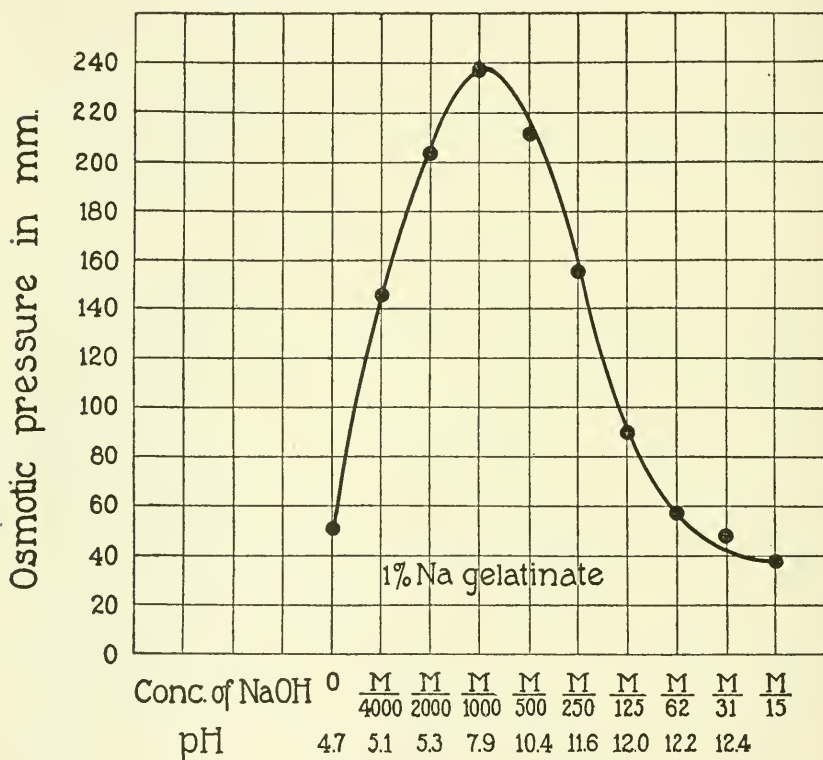


FIG. 1. Curve of osmotic pressure of 1 per cent Na gelatinate solutions contained in collodion bags and surrounded by solutions of NaOH of the same concentration as that in which the isoelectric gelatin was dissolved. Abscissæ are the logarithms of concentration of NaOH in which the isoelectric gelatin was dissolved; below is given the pH of the gelatin solution at the conclusion of the experiment ($4\frac{1}{2}$ hours after beginning). The osmotic pressure rises steeply at first until the pH is about 8.4, and then when more NaOH is contained in solution the osmotic pressure falls again equally steeply with a further increase in concentration of NaOH added.

the absorption of CO_2 from the air and this diminished the concentration of NaOH in the outside and, as a consequence, also in the inside solution.

Fig. 1 represents the osmotic pressure reached after about 5 hours in the various solutions. (At this time the permanent osmotic pressure is generally attained when the solution undergoes no further chemical changes.) The abscissæ of Fig. 1 are the logarithms of the concentration of the NaOH . The row of figures below the figures for the concentration of NaOH is the pH as found in each gelatin solution at the end of the experiment. The ordinates are the osmotic

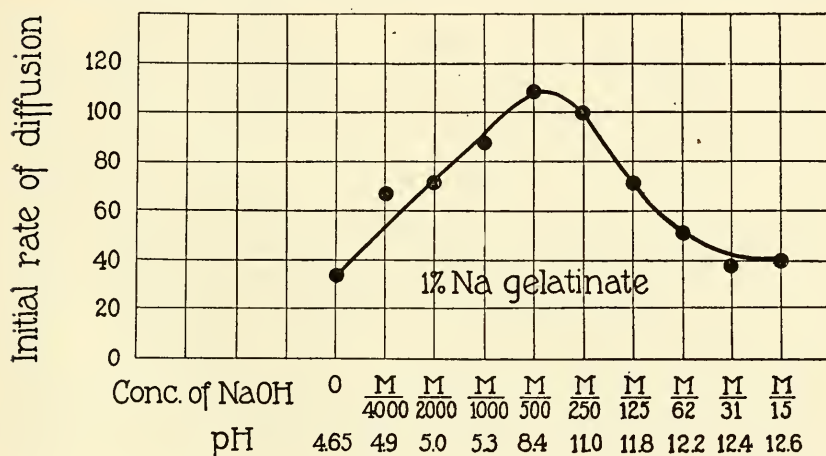


FIG. 2. Curve of initial rate of diffusion of water into 1 per cent gelatin dissolved in the same concentrations of NaOH as those used in Fig. 1, measured by rise of level of liquid in manometer 30 minutes after beginning of experiment. Abscissæ are the concentrations of NaOH , ordinates are the rise of level of liquid in manometer after 30 minutes. The curve is similar to the curve of osmotic pressure. Maximum at $\text{pH} = 8.4$.

pressures expressed in mm. of a column of 1 per cent gelatin solution. The maximum should have been at a pH of about 8.4 (between $M/1,000$ and $M/500$). The figure shows that the osmotic pressure rises first rapidly with an increase in the pH and falls equally rapidly beyond a pH which (from other experiments) we judge to be about 8.4. At a pH of 12.2 the curve is almost as low as it is for isoelectric gelatin.

Fig. 2 shows the influence of the concentration of NaOH upon the initial rate of diffusion of water into the solutions of Na gelatinate. The ordinates represent the height to which the liquid had risen in the manometer 30 minutes after commencement of the experiment. The abscissæ are again the logarithms of the concentration of the NaOH and the pH is that found after 30 minutes. The reader will notice that the critical points in this curve coincide with the critical points in Fig. 1. The curve rises steeply until $\text{pH} = 8.4$ when the maximum is reached, and falls equally steeply until a $\text{pH} = 12.2$ is reached. We have given the reason for this coincidence of the critical points in the curves of Figs. 1 and 2 in a preceding paper.⁵

The question arises, what causes the drop of the two curves when the pH exceeds 8.4? This drop seems to be the same as the drop in the initial rate of diffusion of water into a solution of Na_2SO_4 which occurs when the concentration exceeds $M/256$. When we separate metal gelatinates from distilled water by a collodion membrane, water diffuses into the gelatin solution as if its particles were positively charged; being attracted by the gelatin anion and repelled by the metal ion. Metal gelatinates behave therefore towards the electrification and rate of diffusion of water through collodion membranes like neutral or alkaline solutions of Na_2SO_4 or $\text{Na}_4\text{Fe}(\text{CN})_6$. The analogy can be carried further, since the addition of little salt depresses both the rate at which water will diffuse into the gelatin solution through the membrane as well as the permanent osmotic pressure of the solution.

Fig. 3 shows the rapid fall in the permanent osmotic pressure of a 1 per cent solution of Na gelatinate with a pH of about 8.4 to which various concentrations of a salt, KCl, K_2SO_4 , K_3 citrate, and CaCl_2 , are added. The amounts of salts contained in 100 cc. of gelatin solution were 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of $M/4$. These solutions were put into the collodion bags described, and the collodion bags were put into beakers containing the same salt in the same concentration which had been added to the gelatin solution. Fig. 3 gives the rise in the manometer after 30 minutes and Fig. 4 the osmotic pressure after 6 hours. The curves show the rapid drop

⁵ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 87.

of the initial rate of diffusion, as well as of the permanent osmotic pressure with the increase in the concentration of the salt added. The drop is more rapid when Ca is added than when K is added, as it should be on account of the fact that the particles of water diffusing through the membrane are positively charged.

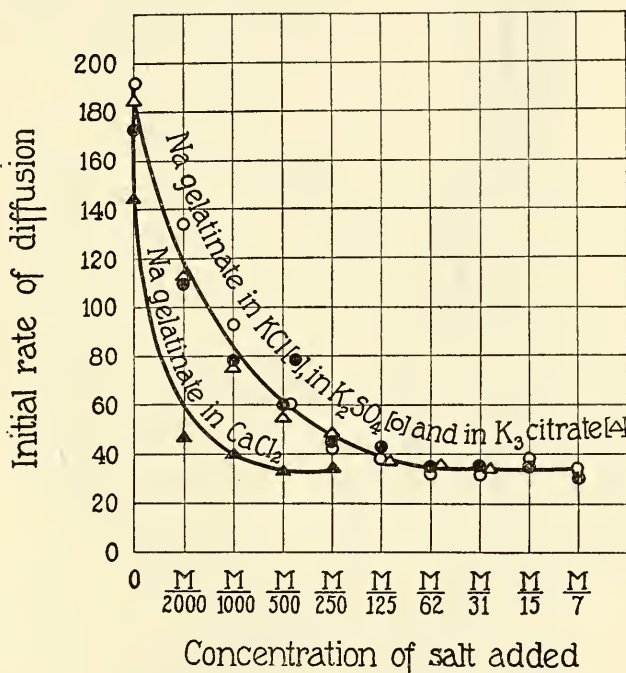


FIG. 3. Depressing effect of the addition of neutral salt to a 1 per cent solution of Na gelatinate of pH about 8.4 upon the initial rate of diffusion of water into the solution during 30 minutes. Abscissæ are the concentration of salt added, ordinates the height of level of liquid in manometer after 30 minutes. The depressing effect of Ca is greater than that of K.

Figs. 5 and 6 show that the depressing effect of the addition of alkali is the same as that of the addition of neutral salt with the same cation, $Ca(OH)_2$ causing a greater depression than KOH . This contradicts the statement current in the literature of colloid chemistry that salts lower and that alkalies raise the osmotic pressure of gelatin solutions.

The influence of the concentration of electrolytes upon the electrification and rate of diffusion of water into gelatin solutions is similar

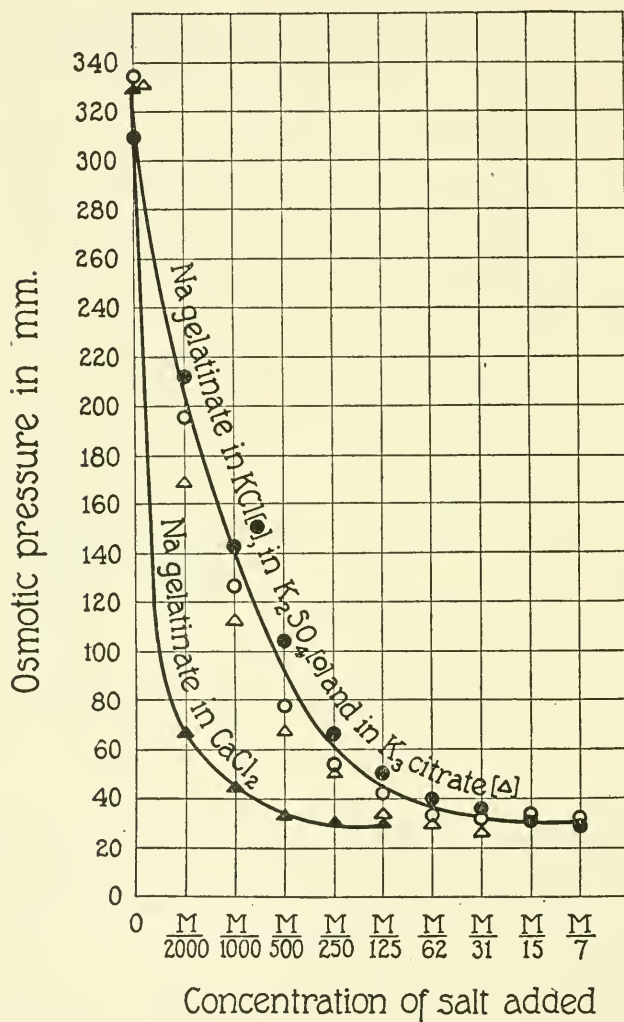


FIG. 4. Depressing effect of the addition of salts to 1 per cent Na gelatinate of pH = 8.4 upon osmotic pressure of solution. Ca depresses more than K.

to the influence of the concentration of electrolytes when we substitute a neutral salt like Na_2SO_4 or $Na_4Fe(CN)_6$ for the solution of metal

gelatinate. It may be well to point out this analogy by making the experimental methods in both cases as much alike as possible.

To solutions of $M/256$ Na_2SO_4 were added the same concentrations of salt as in the gelatin experiments; namely, 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of $M/4$ salt in 100 cc. of solution. These solutions were put into collodion bags and the latter were put into the solutions of the same salts as those in which the Na_2SO_4 was made

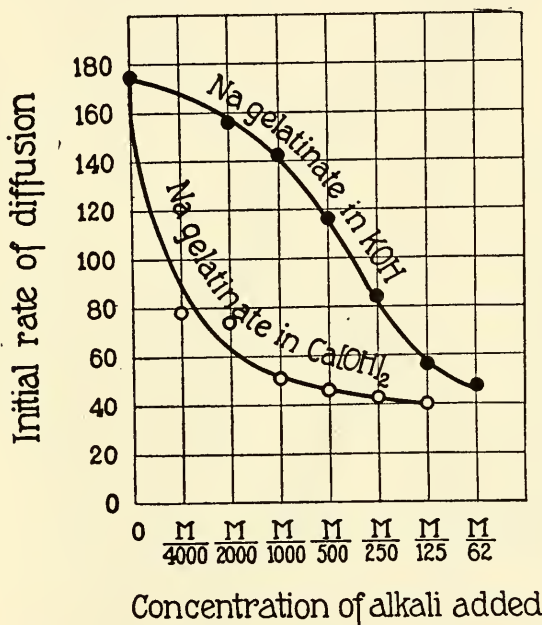


FIG. 5. Depressing effect of the addition of alkali to 1 per cent Na gelatinate of $\text{pH} = 8.4$ upon the initial rate of diffusion of water into the gelatin solution. $\text{Ca}(\text{OH})_2$ depresses more than KOH .

up. This means, that when 100 cc. of the solution in the collodion bag were a mixture of $M/256$ Na_2SO_4 in $M/2,000$ KCl , the outside solution was $M/2,000$ KCl (without Na_2SO_4), and so on. This was done to make the experiments in every point like the gelatin experiments, in which the outside solution also contained the same concentration of salt which was added to the gelatin solution. It was found that the addition of salts and of alkali depresses the initial rate of

diffusion of water into a $M/256$ solution of Na_2SO_4 in the same way as it depresses the initial rate of diffusion of water into a solution of Na gelatinate.

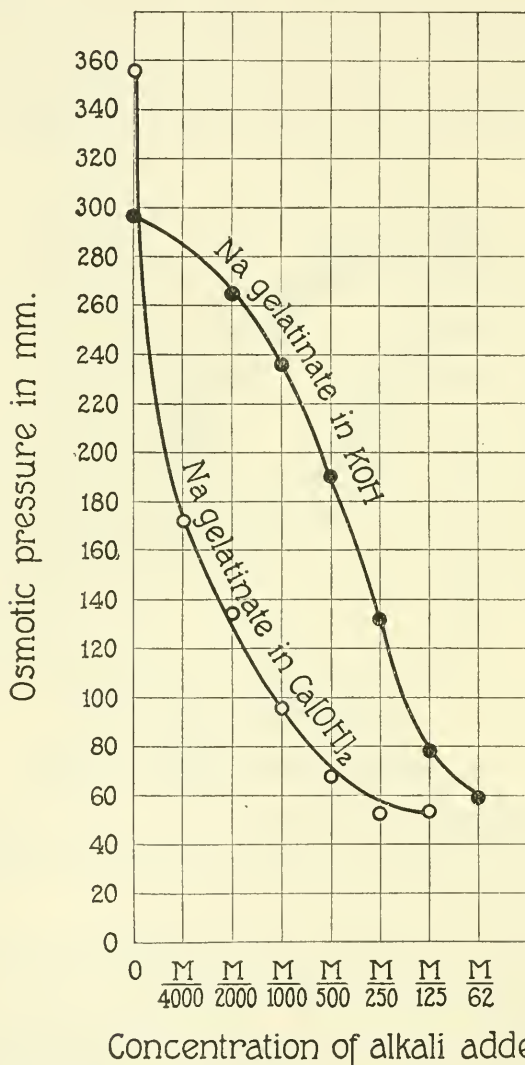


FIG. 6. Depressing effect of the addition of alkali to 1 per cent Na gelatinate of $\text{pH} = 8.4$ upon osmotic pressure of gelatin solution. $\text{Ca}(\text{OH})_2$ depresses more than KOH . The effect is similar to that of addition of neutral salt.

This is illustrated by the experiments represented in Fig. 7. The abscissæ are the concentration of the salt added to the $M/256$ Na_2SO_4 solutions. The electrolytes added were KOH , KCl , and MgCl_2 .

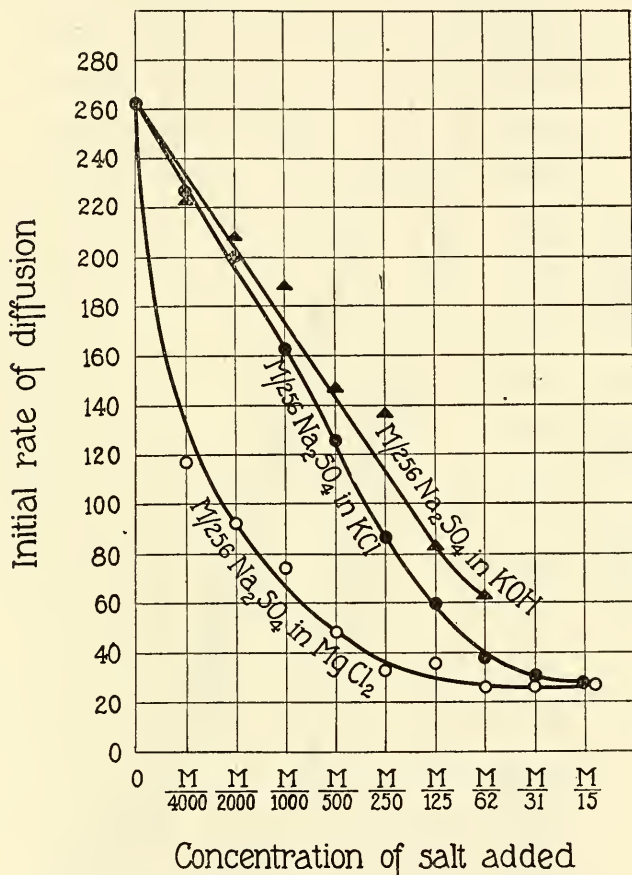


FIG. 7. $M/256$ Na_2SO_4 solutions separated from water by a collodion membrane, showing that the addition of salt or alkali to $M/256$ Na_2SO_4 causes a similar depression upon the initial rate of diffusion of water into the solution of $M/256$ Na_2SO_4 as the addition of salt to a solution of Na gelatin. Mg depresses more than K .

There was little difference in the effect of KOH and of KCl , while the depressing effect of MgCl_2 was considerably greater. *It should be stated that the collodion membranes used in these experiments had not been treated with gelatin.*

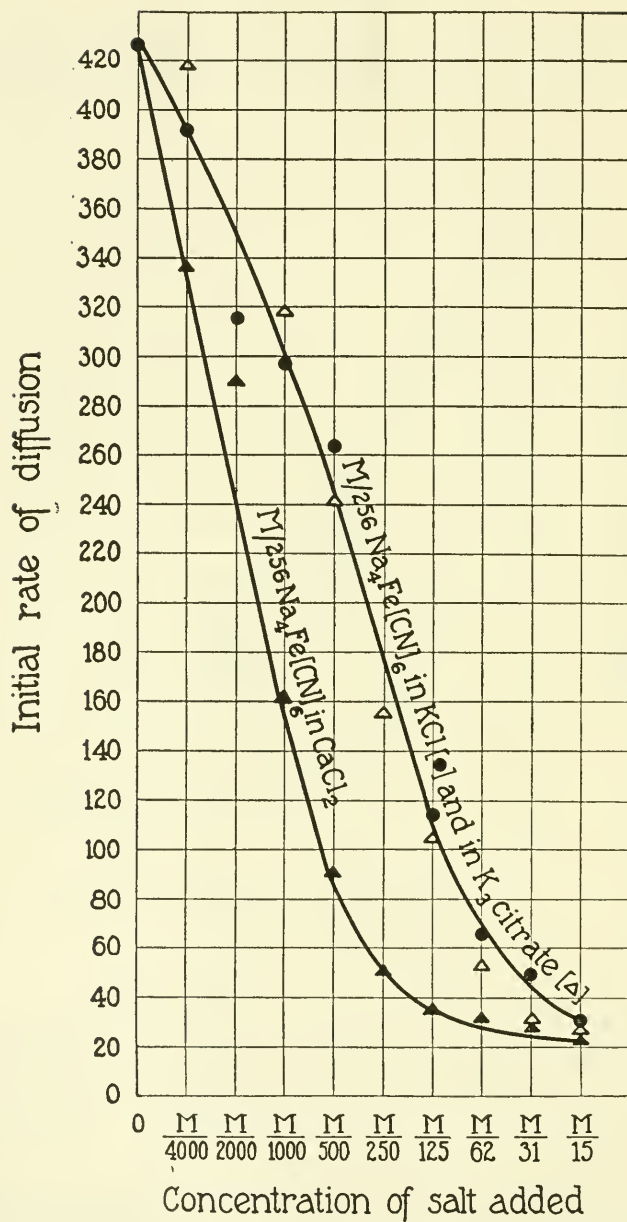


FIG. 8. $M/256 Na_4Fe(CN)_6$ separated from water by a collodion membrane, showing the depressing effect of the addition of salt to the solution upon the initial rate of diffusion of water into the solution.

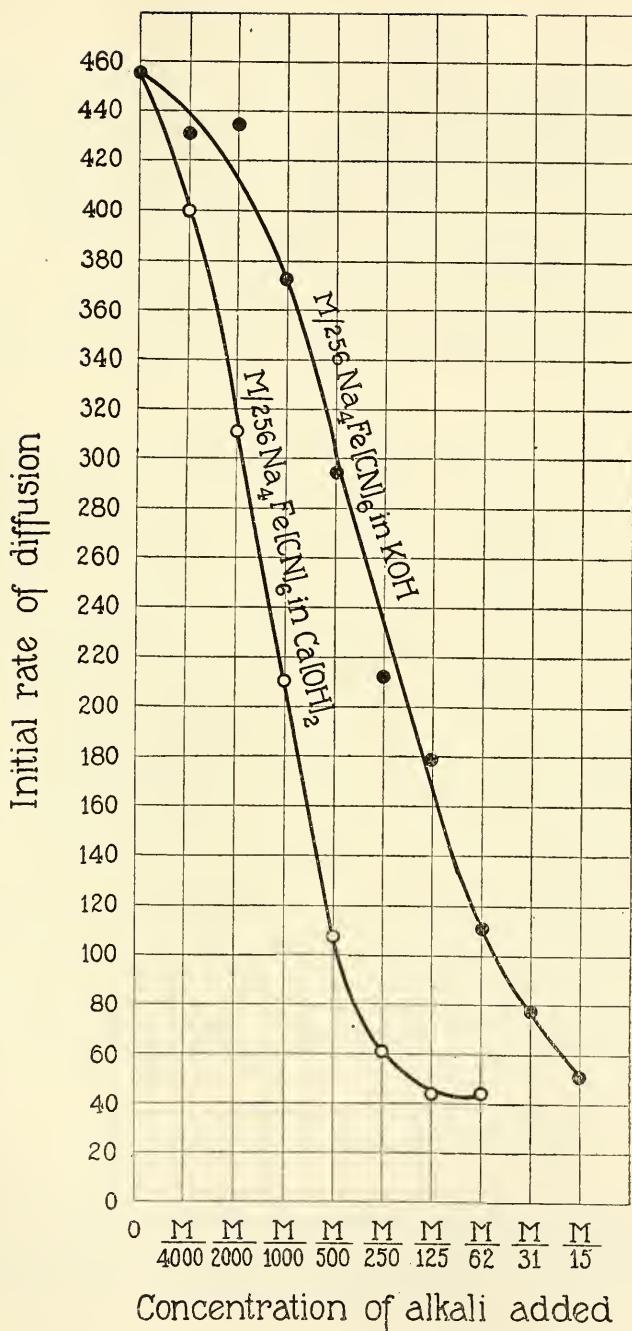


FIG. 9. Showing the same depressing effect of the addition of alkali to $M/256 \text{ Na}_4\text{Fe(CN)}_6$.

These experiments can be repeated with any other neutral or alkaline salt with univalent cation. Figs. 8 and 9 show the depressing effect of the addition of neutral salts (Fig. 8) or of alkalies (Fig. 9) to a solution of $M/256 \text{ Na}_4\text{Fe}(\text{CN})_6$. Again the influence of the valency of the cation and the identity of the effects of alkalies and neutral salts with the same cation are manifest.

In all these experiments in which the particles of water were positively charged the depressing effect of the addition of salt was greater when the cation of the salt was bivalent than when it was monovalent.

Such experiments have been made with a large number of salts, all yielding the same result; namely, that the depressing effect of the addition of increasing concentrations of alkalies and neutral salts upon the attraction of water by gelatin solutions is paralleled by the influence of the addition of increasing concentrations of salts upon the attraction of water by solutions of salts, in the presence of which water is positively charged. This raises the question whether or not the depressing influence of high concentrations of electrolytes upon the osmotic pressure of gelatin is necessarily connected with the colloidal character of gelatin or whether it is based upon a much more general property of matter; namely, the influence of electrolytes upon the electrification and the rate of diffusion of water through membranes. We only wish to point out this possibility without deciding definitely.

It may suffice to point out that the influence of the concentration and the valency of electrolytes on the osmotic pressure of gelatin solutions can be demonstrated equally well for the phenomenon of swelling and the curves representing this influence are similar to those given in this paper for the influence on the osmotic pressure.

III. Negatively Charged Particles of Water.

When we separate solutions of gelatin-acid salts from pure water by a collodion membrane, the particles of water diffusing through the membrane act as if they were negatively charged. That this is so can be demonstrated by experiments on electrical endosmose.

When acid, *e.g.* HCl, is added to isoelectric gelatin, part of the acid combines with gelatin to form gelatin chloride and part of the acid remains free. There is a chemical equilibrium between isoelectric gelatin, gelatin chloride, and free hydrochloric acid. As long as the pH of the gelatin chloride solution does not exceed 3.3 (*i.e.* as long as the pH varies between 4.7 and 3.3) the osmotic pressure of gelatin chloride increases with an increase in the concentration of the acid. As soon, however, as the pH reaches 3.3, the osmotic pressure of gelatin chloride diminishes again with increasing concentration of acid.

Fig. 10 may serve as an illustration. To each of a series of doses of 1 gm. of isoelectric gelatin were added 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 HCl, water was added, the gelatin melted by heating to about 40°C., and enough water was added to make the volume of the solution 100 cc., so that each solution contained 1 per cent gelatin. This solution was put into collodion bags closed by a rubber stopper perforated with a glass tube serving as a manometer. The collodion bag was put into a beaker containing 350 cc. of the same concentration of acid in water as that originally added to the gelatin. Thus the gelatin solution to which 0.2 cc. of M/4 HCl was added per 100 cc. of solution was immersed in a HCl solution containing 0.2 cc. of M/4 HCl in 100 cc. of water. This outside solution was a little more concentrated than the concentration of the free acid inside, since part of the acid added to the gelatin entered into combination with the latter. This difference was equalized by the diffusion of some of the outside acid into the gelatin solution, thus slightly lowering the original pH.

The upper curve (Fig. 10) shows that the osmotic pressure of the solution (measured after 20 hours) rises with an increase of the hydrogen ion concentration until it reaches a maximum at pH about 3.4 or 3.3, and that with a further rise in the amount of free acid the osmotic pressure of the solution falls until at pH 1.75 the osmotic pressure of the gelatin solution is almost as low as it is near the isoelectric point.

The lower curve in Fig. 10 represents the velocity of diffusion of water into the gelatin solution measured by the height of the column of liquid in the manometer after 30 minutes. The maximum and the two minima of the curve coincide with those of the upper curve

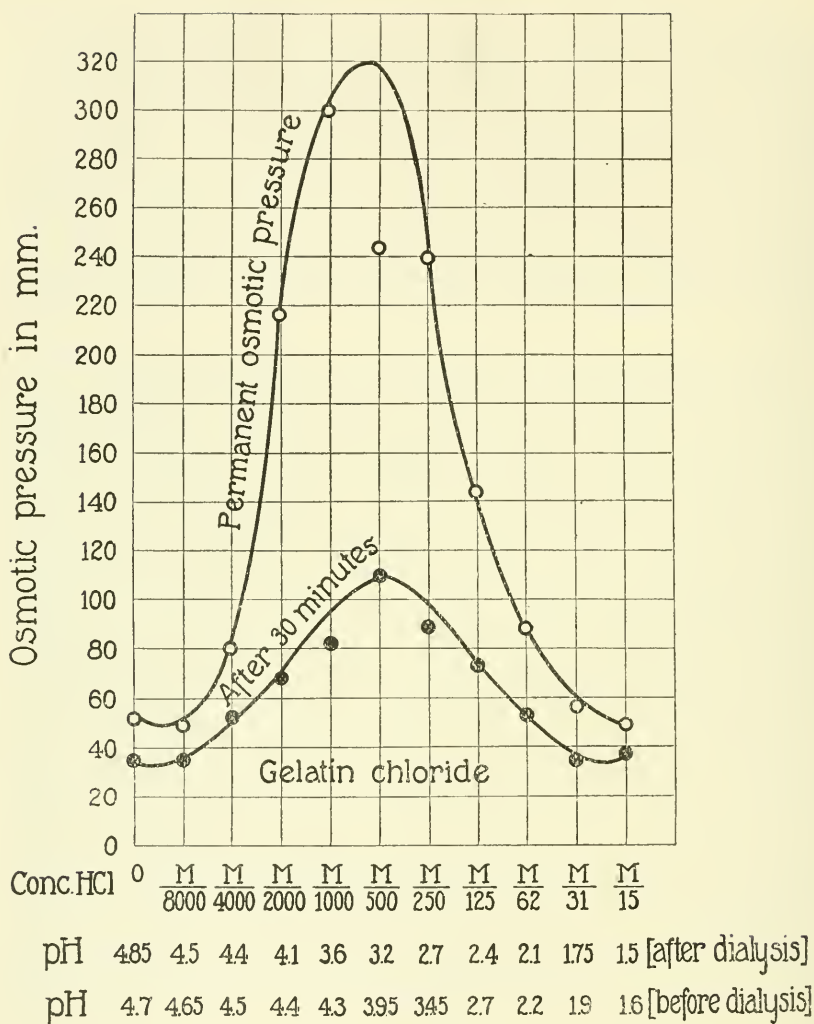


FIG. 10. 1 per cent isoelectric gelatin solutions dissolved in increasing concentrations of HCl. Abscissae are the concentrations of HCl in which the gelatin is dissolved, with the pH at the end and at the beginning of experiment. Ordinates of upper curve represent the final osmotic pressure (after 20 hours); ordinates of lower curve represent the initial rate of diffusion of water into solution. Maximum in both cases at pH 3.3. Showing the depressing effect of the further addition of HCl when pH 3.3 is reached.

representing the permanent osmotic pressures of the various gelatin solutions.

When we select gelatin chloride with about the maximal osmotic pressure, *i.e.* gelatin chloride with a pH of 3.5, and add various

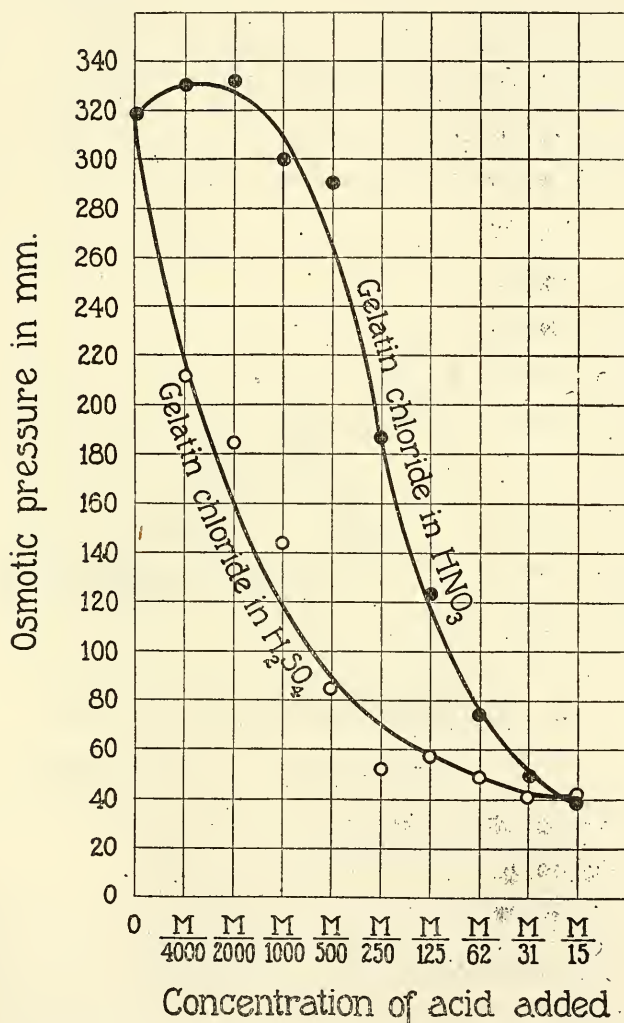


FIG. 11. Showing depressing effect of addition of HNO_3 and H_2SO_4 to 1 per cent gelatin chloride upon osmotic pressure. Depressing effect of SO_4 greater than that of HNO_3 .

concentrations of another acid to it, we notice a similar depression. Fig. 11 gives the effect of the addition of different quantities of HNO_3 and of H_2SO_4 to 1 gm. of gelatin of pH 3.3; 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 acid were contained in 100 cc. of gelatin solution of pH 3.5. The outside solution contained the same concentration of acid. The depressing effect of HNO_3 was like that of HCl , and the depressing effect of H_2SO_4 was greater. Fig. 12 shows that the depressing influence of the addition of acid is manifested in a similar way in the influence of the concentration of acid upon the initial diffusion of water into the gelatin solution.

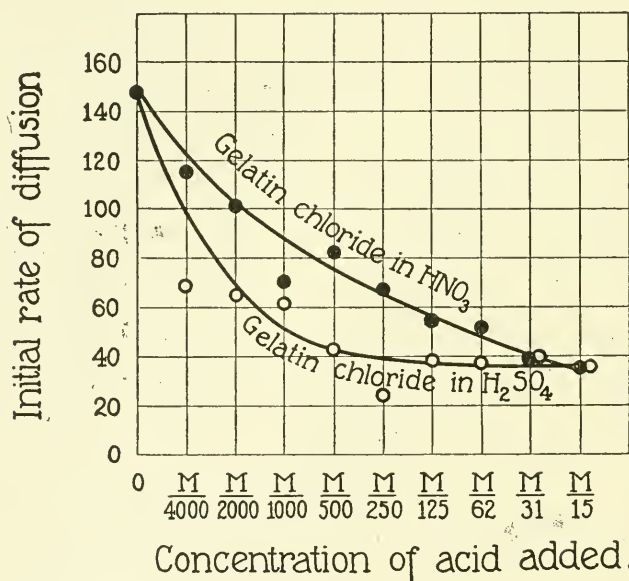
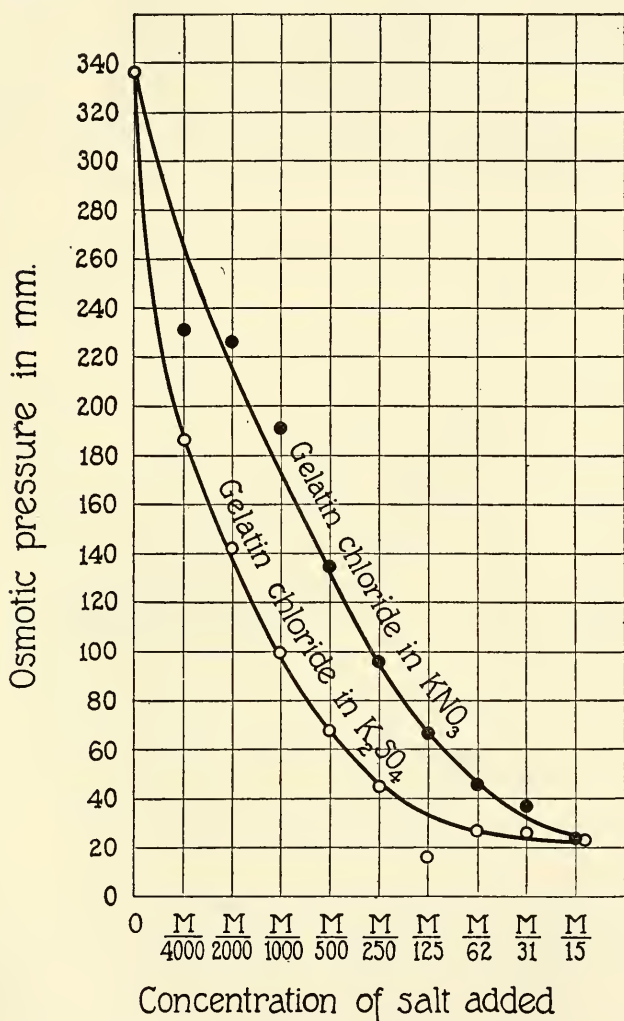


FIG. 12. The same effect of the addition of acid upon the initial rate of diffusion of water into the gelatin chloride solution.

If we add neutral salt, *e.g.* KNO_3 or K_2SO_4 , to a 1 per cent gelatin solution with a pH = 3.5, taking care that the pH of the solution is not altered by the addition of salt, the depressing effect is about the same as when we add acid. To doses of 1 gm. isoelectric gelatin of pH 3.5 were added 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 cc. of M/4 KNO_3 or K_2SO_4 , and enough water was added to make the volume 100 cc. The pH remained the same in all solutions.

The outside solutions had the same pH as the solution in the colloid bag and contained also the same concentration of potassium



FIGS. 13 and 14. Depressing effect of addition of salt to 1 per cent gelatin chloride pH = 3.3. SO₄ depresses more than NO₃.

salt as was added to the gelatin. The curves in Figs. 13 and 14 show that the depressing effect of an addition of KNO₃ is about the same as the addition of an equal amount of HNO₃, and that the addition

of K_2SO_4 has about the same depressing effect as the addition of H_2SO_4 .

When gelatin-acid salts, *e.g.* gelatin chloride, are separated from distilled water or a salt solution of the same pH by a collodion bag, water diffuses through the membrane in the form of negatively charged particles which are attracted by the gelatin cation and repelled by the anion. When we wish to replace the gelatin solution by a crystalloidal electrolyte in the presence of which water diffuses

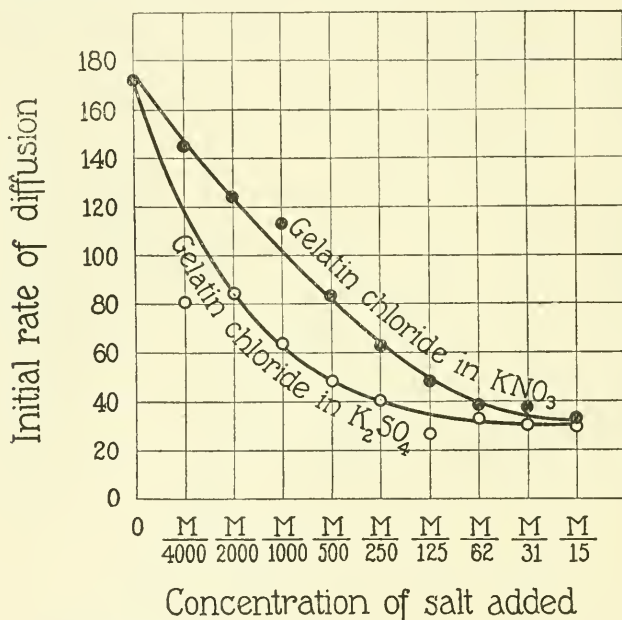


FIG. 14.

through the membrane in the form of negatively charged particles, we have to fulfill two conditions. First, we must use as electrolytes salts with trivalent (or tetravalent) cation, *e.g.* $CeCl_3$ or $AlCl_3$, or if we wish to use salts with monovalent or bivalent cation the salts must be rendered sufficiently acid; *e.g.*, pH = 4.0 or less. Second, the collodion membrane must previously receive a treatment with gelatin. The gelatin will be rinsed out, but some gelatin obviously adheres to the wall. As I shall show in another paper this treatment

of the collodion membrane with a protein is necessary since otherwise water will diffuse through the collodion membrane in the form of positively charged particles and then the case is no longer comparable with that of gelatin-acid salts. This gelatin treatment of the collodion membrane is not required when we wish to experiment with positively charged particles of water, since water assumes practically always a positive charge when in contact with a collodion membrane free from gelatin, even in comparatively high concentrations of acid.

If we use membranes which had contained a 1 per cent gelatin solution over night but were then freed from the gelatin by a number of washings with warm water, and if we substitute for the gelatin-acid salt a crystalloidal salt which causes water to be charged negatively in such gelatin-treated membranes, *e.g.* $M/512 \text{ Al}_2\text{Cl}_6$, we notice the same depressing effect of an increase of the concentration of electrolytes on the initial rate of diffusion of water into the solution as was described in the case of the solutions of gelatin-acid salts. When we separate a collodion bag filled with $M/512 \text{ Al}_2\text{Cl}_6$ (which causes water to be charged negatively) and put it into H_2O , the level of liquid in the manometer will rise to a height of about 300 mm. in 20 minutes. When we add varying quantities of a neutral salt to the $M/512$ solution of Al_2Cl_6 , and put the same concentration of salt into the outside beaker containing the water—in order to eliminate the direct effect of the salt added upon the rate of diffusion—we notice that the rate of diffusion of water will diminish the more rapidly the more salt we add. This is illustrated in Fig. 15. NaNO_3 and CaCl_2 have about the same depressing effect, showing that the depression is not due to the influence of the cation; while SO_4 and still more citrate depress much more powerfully than the chlorides, showing that the depression is due to the influence of the increasing concentration of the anion upon the negatively charged particles of water.

Similar experiments were made with solutions of CaCl_2 which were rendered sufficiently acid in order to cause the water to be charged negatively. If we select $M/128$ solutions of such acidulated CaCl_2 as a substitute for gelatin-acid solutions, we find that the addition of acid as well as of neutral salt causes a depression of the influence of

the acidulated CaCl_2 solution upon the initial rate of diffusion of water into the solution.

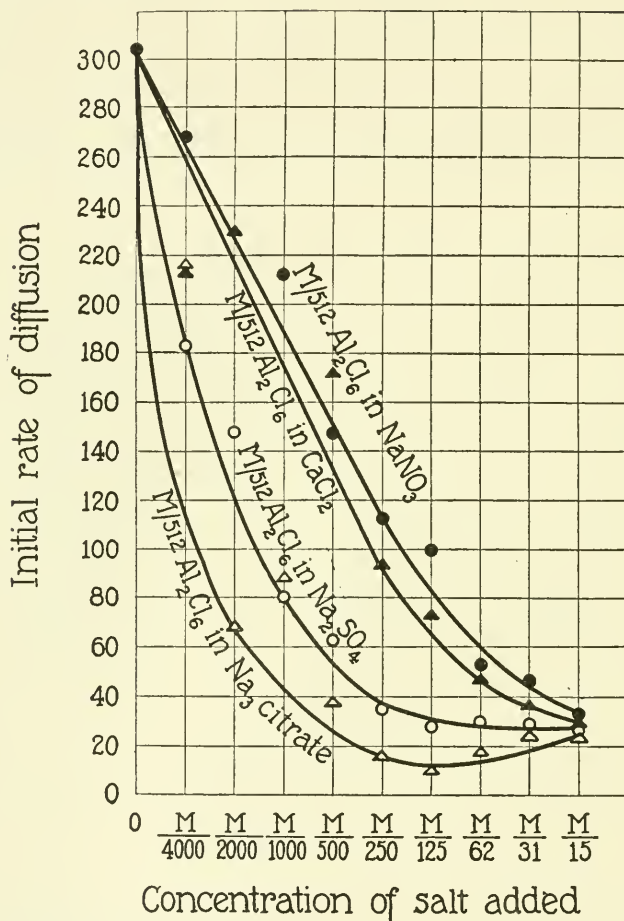


FIG. 15. $\text{M/512 Al}_2\text{Cl}_6$ separated from water by collodion bag. Depressing effect of addition of salt to $\text{M/512 Al}_2\text{Cl}_6$ upon the initial rate of diffusion of water into the bag. SO_4 depresses more than NO_3 or Cl .

In all the experiments mentioned the electrolyte was added not only to the inside but also to the outside solution. It is hardly necessary to state that the depressing effect is also well marked if the electrolyte is added only to the solution inside the collodion bag, while on the outside is distilled water.

These facts, then, show that the depressing effect of the addition of electrolyte upon the osmotic pressure of gelatin solutions (or colloidal solutions in general) is paralleled by the effect the addition of electrolyte has upon the initial rate of diffusion from water into solution through a collodion membrane, when we substitute a crystalloidal electrolyte for the gelatin salt.

SUMMARY.

1. When a 1 per cent solution of a metal gelatinates, *e.g.* Na gelatinates, of $\text{pH} = 8.4$ is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain rate which can be measured by the rise of the level of the liquid in a manometer. When to such a solution alkali or neutral salt is added the initial rate with which water will diffuse into the solution is diminished and the more so the more alkali or salt is added. This depressing effect of the addition of alkali and neutral salt is greater when the cation of the electrolyte added is bivalent than when it is monovalent. This seems to indicate that the depressing effect is due to the cation of the electrolyte added.

2. When a neutral $m/256$ solution of a salt with monovalent cation (*e.g.* Na_2SO_4 or $\text{K}_4\text{Fe}(\text{CN})_6$, etc.) is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain initial rate. When to such a solution alkali or neutral salt is added, the initial rate with which water will diffuse into the solution is diminished and the more so the more alkali or salt is added. The depressing effect of the addition of alkali or neutral salt is greater when the cation of the electrolyte added is bivalent than when it is monovalent. This seems to indicate that the depressing effect is due to the cation of the electrolyte added. The membranes used in these experiments were not treated with gelatin.

3. It can be shown that water diffuses through the collodion membrane in the form of positively charged particles under the conditions mentioned in (1) and (2). In the case of diffusion of water into a neutral solution of a salt with monovalent or bivalent cation the effect of the addition of electrolyte on the rate of diffusion can be explained on the basis of the influence of the ions on the electrifica-

tion and the rate of diffusion of electrified particles of water. Since the influence of the addition of electrolyte seems to be the same in the case of solutions of metal gelatinates, the question arises whether this influence of the addition of electrolyte cannot also be explained in the same way, and, if this be true, the further question can be raised whether this depressing effect necessarily depends upon the colloidal character of the gelatin solution, or whether we are not dealing in both cases with the same property of matter; namely, the influence of ions on the electrification and rate of diffusion of water through a membrane.

4. It can be shown that the curve representing the influence of the concentration of electrolyte on the initial rate of diffusion of water from solvent into the solution through the membrane is similar to the curve representing the permanent osmotic pressure of the gelatin solution. The question which has been raised in (3) should then apply also to the influence of the concentration of ions upon the osmotic pressure and perhaps other physical properties of gelatin which depend in a similar way upon the concentration of electrolyte added; *e.g.*, swelling.

5. When a 1 per cent solution of a gelatin-acid salt, *e.g.* gelatin chloride, of pH 3.4 is separated from distilled water by a collodion membrane, water will diffuse into the solution with a certain rate. When to such a solution acid or neutral salt is added—taking care in the latter case that the pH is not altered—the initial rate with which water will diffuse into the solution is diminished and the more so the more acid or salt is added. Water diffuses into a gelatin chloride solution through a collodion membrane in the form of negatively charged particles.

6. When we replace the gelatin-acid salt by a crystalloidal salt, which causes the water to diffuse through the collodion membrane in the form of negatively charged particles, *e.g.* M/512 Al_2Cl_6 , we find that the addition of acid or of neutral salt will diminish the initial rate with which water diffuses into the M/512 solution of Al_2Cl_6 , in a similar way as it does in the case of a solution of a gelatin-acid salt.

QUANTITATIVE LAWS IN REGENERATION. I.

By JACQUES LOEB.

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(Received for publication, November 22, 1919.)

Regeneration consists in the growth of resting cells or buds as a consequence of the removal of parts of the body. Two problems are, therefore, involved in this phenomenon; namely, first, the quantity of growth which is measured by the mass of the regenerated organs, and, second, the problem of inhibition, or the fact that regeneration or growth of the resting cells or buds does not take place without removal of part of the body.

I. The Law of Direct Proportionality between the Mass of Sister Leaves of Bryophyllum and the Mass of Roots and Shoots Produced by Them.

The writer had already shown that equal masses of sister leaves of *Bryophyllum calycinum* when isolated from the rest of the plant produce in equal time equal masses of shoots under equal conditions of temperature, moisture, and illumination.¹ The following measurements show that the same law holds also for the production of roots. In order to be able to measure the mass of roots correctly, it was necessary to let the roots develop in water. Part of a leaf was dipped into water, and in the wetted notches shoots and roots grew out rapidly, thereby preventing the growth of roots and shoots from the non-submerged notches of the leaf. In order to get accurate measurements of root production it was necessary to determine the dry weight of the roots. In addition, the dry weight of the leaves and of the shoots was also determined. The experiments lasted usually from 4 to 5 weeks in order to get larger masses of roots and to diminish the error in determining the quantity of regeneration. The organs were dried in an oven at a temperature of between 100° and 110°C. for about 24 hours.

¹ Loeb, J., *Bot. Gaz.*, 1918, lxxv, 150.

Five pairs of sister leaves were suspended sidewise, the lower edge dipping in water, and roots and shoots formed at the lower edges only. Table I gives the dry weights of the leaves, of the shoots, and of the roots at the end of the experiment. The figure in parentheses, behind the dry weight of the shoots, expresses the number of shoots formed. The first horizontal row gives the dry weights of these organs for one set of five leaves (Set I), the second horizontal row gives the dry weights of these organs for the five sister leaves (Set II). The third horizontal row gives the ratio of the dry weights of the two sets of organs. The masses of the two sets of sister leaves were almost alike, and the ratio of the two masses was 1.02. According to the law expressed in the writer's former publications the

TABLE I.
Duration of Experiment 34 Days.

	Dry weight of		
	Leaves.	Shoots.	Roots.
	gm.	gm.	gm.
Set I.....	1.755	0.247 (14)	0.113
" II.....	1.718	0.247 (15)	0.120
Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$	1.02	1.00	0.94

mass of shoots and of roots produced by the two sets of leaves should also be alike (within the limits of accuracy of these experiments). The ratio of the two masses of shoots and roots should, therefore, have been approximately 1.00.

The figures of the third row show that the ratio of the dry weights of the two sets of shoots is exactly 1.00 and the ratio of the two sets of roots is 0.94. *The two sets of sister leaves having equal mass produced, during the same time and under equal conditions of temperature, moisture, and illumination, equal masses of shoots and of roots.*

In the next experiment one set of six isolated leaves (Set II) remained intact while the mass of each leaf of the second set was reduced to approximately one-half by cutting away one side of each leaf (Set I) (Fig. 1). The masses of the two sets of leaves were

therefore no longer equal but had approximately the ratio of about 1:2. It was to be expected that the dry weights of the shoots and roots produced by the two sets of leaves should also be in the ratio of about 1:2, and this was the case (within the limits of the possible accuracy of such experiments) (Table II). The ratio of the dry weights of the two sets of leaves was 0.54, the ratio of the weights

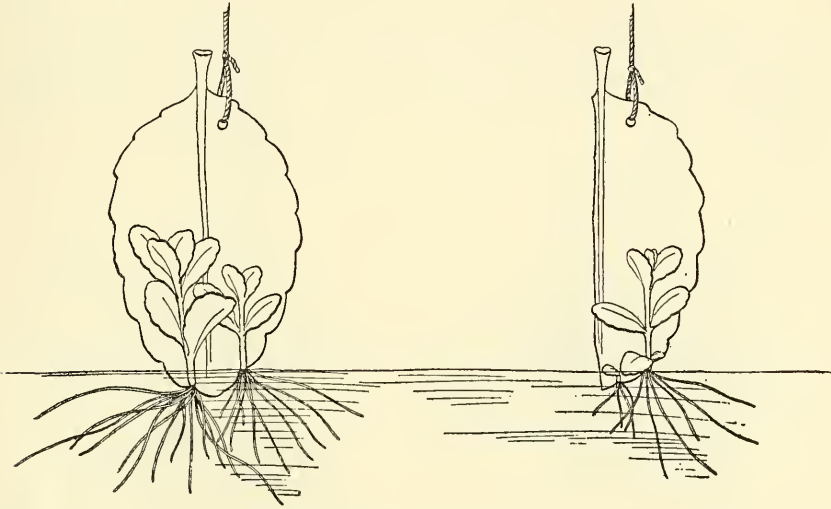


FIG. 1. Root and shoot formation in whole leaf and in sister leaf (from which the left half is cut off) dipping with their apices in water. Root and shoot formation only in the wetted parts of leaf. Diagrammatic.

TABLE II.

Duration of Experiment 30 Days.

	Dry weight of		
	Leaves.	Shoots.	Roots.
	gm.	gm.	gm.
Set I (six half leaves).....	1.245	0.174 (10)	0.054
" II (" whole ").....	2.300	0.283 (16)	0.092
Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$	0.54	0.61	0.59

of shoots produced by them was 0.61, and the ratio of roots produced was 0.59. The tips of the two sets of leaves dipped in water and roots and shoots developed only in the submerged notches (Fig. 1).

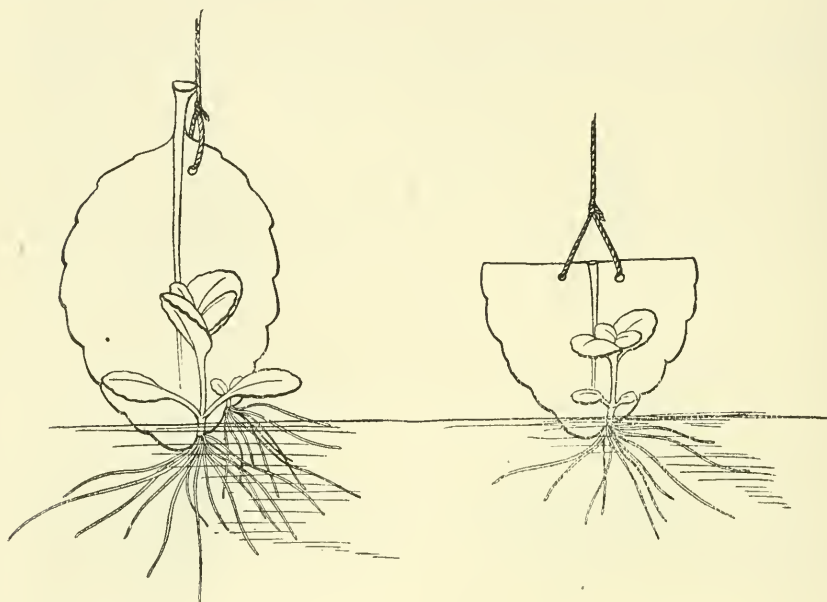


FIG. 2. Shoot and root formation in whole leaf and in sister leaf (from which the basal part is cut off), both dipping with their apices in water. Duration of experiment 34 days. After nature.

TABLE III.

Duration of Experiment 33 Days.

	Dry weight of		
	Leaves.	Shoots.	Roots.
	gm.	gm.	gm.
Set I (six reduced leaves).....	0.794	0.156 (11)	0.043
" II (" whole ").....	2.127	0.343 (19)	0.116
Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$	0.37	0.45	0.37

In the next experiment one set of six leaves remained intact (Set II) while more than half of the basal part of the set of sister leaves (Set I) was cut off (Fig. 2). The tips of the leaves dipped into water. Again the ratio of the mass of shoots and roots regenerated was, within the limits of the accuracy of the experiments, the same as the ratio of the masses of the two sets of leaves. It was 0.37 for the leaves and exactly the same number for the roots, while it was slightly larger, 0.45, for the shoots (Table III).

In the next set, the leaves were suspended sidewise, their lower edges dipping in water (Fig. 3). In both sets of leaves the upper edge was cut off; in one set the lower edge with the exception of one

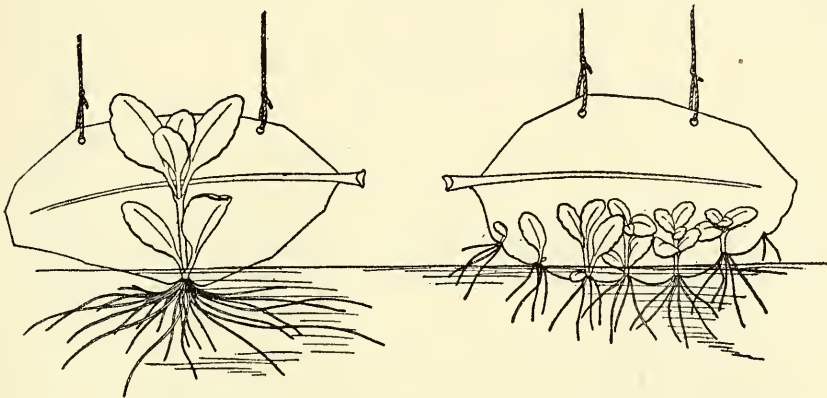


FIG. 3. Leaves suspended sidewise. The upper edge is cut off in both leaves. In leaf to left all notches except one removed, in leaf to right all notches on the lower side preserved. Duration of experiment 34 days. After nature.

or two notches was cut off (Set I), while in the other set (Set II) all the notches of the lower edge were left intact. This was done to make the number of shoots produced by both sets unequal and to show that the general law enunciated is independent of the number of shoots produced. Thus, as Table IV shows, the leaves of Set I produced six and the leaves of Set II produced sixteen shoots, yet the mass of the two sets of shoots was almost the same, though it is not impossible that when more shoots develop in one leaf the available material furnished by the leaf can be utilized more fully than when all the material goes into one shoot.

On the basis of these and of the writer's earlier experiments we can enunciate the following law.

Equal masses of sister leaves of *Bryophyllum calycinum* produce in equal time, under equal conditions of temperature, moisture, illumination, and aeration, approximately equal masses of roots and shoots, regardless of the number of shoots or roots formed (except that a moderate number of shoots may possibly permit a more complete utilization of the material furnished by the leaf than if only one shoot is formed). When the masses of two sister leaves are unequal, the masses of shoots and roots produced by them are directly proportional to the masses of the leaves.

TABLE IV.

Duration of Experiment 33 Days.

	Dry weight of		
	Leaves.	Shoots.	Roots.
	gm.	gm.	gm.
Set I. Five leaves with one or two notches.....	1.810	0.248 (6)	0.106
" II. " sister leaves with more "	1.778	0.270 (16)	0.121
Ratio $\frac{\text{dry weight I}}{\text{dry weight II}}$	1.02	0.92	0.88

II. Ratio of the Mass of Shoots to the Mass of Roots Produced by a Leaf Dipped in Water.

When we dip a leaf with part of its edge into water, shoots and roots form as a rule only in the wetted part of the leaf. It was of importance to determine the ratio of dry weight of roots to the dry weight of shoots produced in such cases. In the examples already quoted this ratio was never less than one-third and never more than one-half. The average taken from a large number of experiments gave the ratio of the dry weight of roots to that of shoots under such conditions as 0.42. This figure will be used in the experiments of the next chapter.

III. The Inhibitory Influence of the Stem on Shoot Production in the Leaf of Bryophyllum calycinum.

Only the leaf isolated from the stem is capable of forming shoots; it suffices as a rule to leave a piece of stem connected with the leaf to cause retardation or inhibition of the growth of the dormant buds in the notches of a leaf.² There are two possible reasons for the inhibitory action of the stem on the shoot and root formation in the leaf. The material available for root and shoot formation in the leaf either flows naturally into the stem and hence the buds in the notches of the leaf cannot grow out on account of lack of material for growth;³ or second, the stem sends into the leaf a substance preventing the growth of the notches in spite of the fact that the material needed for the growth of the dormant buds in the notches of the leaf is available.

Only quantitative experiments allow us to decide between the two possibilities. If the assumption is correct that the leaf normally sends the material which can be utilized for the growth of shoots and roots into the stem, the stem in connection with a leaf should gain in weight and this gain should be equal to the mass of shoots and roots the same leaf would produce when separated from the stem. If the other possibility is correct, and if we are dealing with the effect of inhibitory substances sent into the leaf, no such increase in the dry weight of the stem need occur. My experiments give a clear answer in favor of the first possibility; namely, that the inhibitory effect of the stem upon the shoot and root production in the leaf is due to the fact that the material in the leaf which could be utilized for shoot and root formation flows normally into the stem, as long as the leaf is connected with the stem and the sap flow is not interrupted; while when the leaf is separated this material becomes available for the growth of shoots and roots in the leaf, and the inhibition ceases. Of course, we cannot determine directly which mass of shoots and roots the inhibited leaf would have produced if

² This inhibition cannot be permanent since before the falling off of the leaf the sap flow between leaf and stem will cease and this will have the same effect as the cutting off of the leaf.

³ Loeb, J., *Ann. Inst. Pasteur*, 1918, xxxii, 1.

its regeneration had not been inhibited by the stem, but we can determine this quantity indirectly, by measuring the quantity of shoots and roots produced by the sister leaf detached from the stem, since we know that two sister leaves of equal size produce equal masses of shoots and roots in equal time and under equal conditions.

Our method of procedure was as follows. Pieces of stem possessing one node with two healthy leaves of equal size were cut out (Fig. 4). The stem was divided lengthwise as accurately as possible into two equal pieces, b and b_1 , by a cut between the base of the petioles of the two leaves, so that each leaf was connected with one-half piece of stem of equal mass. One leaf (a_1) remained in connection with its piece of half stem (b_1), while the other leaf (a) was detached from its piece (b). The dry weight of this detached piece of half stem b was determined immediately at the beginning of the experiment.

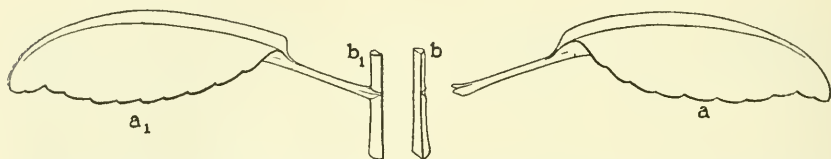


FIG. 4. Diagram to illustrate experiments in which the stem is cut lengthwise.

At the end of the experiment the other piece of half stem b_1 was detached from its leaf and its dry weight was determined. By comparing the dry weight of b_1 with that of b (detached at the beginning of the experiment) it was possible to ascertain how much material the piece b_1 had received from the leaf during the time the experiment lasted. Both leaves, the one detached from the stem as well as the one left in connection with the stem, were suspended in a moist aquarium, the apex of each leaf dipping in water. The detached leaves formed shoots and roots very rapidly, while the leaves in connection with their half pieces of stem formed practically no shoots and roots.⁴

⁴ The inhibitory power of a piece of stem on the shoot formation in the leaf increased within certain limits with the mass of the stem, but not in direct proportion. It was also very obvious that equal masses of stem suppressed the

We selected for each experiment twelve pairs of sister leaves and care was taken that the leaves were healthy, not too large, and of equal size. We made sure that the fresh weight of each set of leaves with their piece of half stem was approximately like that of the set of sister leaves (Table V).

The difference in one set is not more than 3 per cent and is equalized by the fact that six sets of different experiments were made. We proceeded then as follows. The dry weight of b (Fig. 4) was ascertained immediately. Then the leaf a (detached from its stem) and the leaf a_1 (in connection with its piece of half stem) were suspended in the same aquarium dipping with their apices into water. In the leaves a (without stems), roots and shoots formed soon, while none, or practically none, formed in leaves a_1 .

TABLE V.

No. of experiment.	Fresh weight of	
	$a + b$	$a_1 + b_1$
	gm.	gm.
1	28.240	29.103
2	36.625	37.175
3	40.930	39.640

After several weeks the shoots were cut off, their dry weight was determined, and the piece of stem b_1 was also detached from its leaf and its dry weight determined. Let a be the dry weight of shoots and roots formed by the leaves detached from their stem; let a_1 be the dry weight of shoots and roots formed by the leaves or in the axil of the leaves connected with their stem; let b be the dry weight

shoot formation the more completely the smaller the leaf. When the leaf is large it furnishes more material than the cells of a small piece of stem can absorb. It is also of interest that the inhibitory power of a piece of stem on shoot formation in a leaf is smaller in an old leaf than in a young leaf nearer the apex. This influence of age of the leaf is probably connected with the fact that in the oldest leaf the flow of sap into the leaf is more or less incomplete; this interruption of sap flow may possibly be the first step in the histological changes at the base of the leaf which cause it to fall off. In our experiments conditions were selected in such a way as to make the inhibition of shoot and root formation in Leaf a_1 practically complete.

of the piece of half stem at the beginning, b_1 the dry weight of the piece of half stem at the end; then we should expect the following relation to hold (within the limits of accuracy of the experiments).

$$a - a_1 = b_1 - b$$

Six sets of experiments (each with twelve pairs of leaves) were made in which the dry weights of the shoots and of the pieces of stem were determined. The stems were either 2, 4, or 8 cm. long, and with this length of stem the formation of shoots on the pieces connected with the stem was almost completely suppressed. The weights of the roots were not determined but we know that on the average the dry weight of the roots formed by the leaves under the

TABLE VI.

No. of experiment.	Duration of experiment.	a	$a_1 + b_1$	b	$a_1 + b_1 - b$
	<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
1	28	0.141	0.384	0.176	0.208
2	28	0.172	1.1095	0.825	0.284
3	28	0.308	0.7348	0.3846	0.350
4	29	0.216	0.496	0.159	0.337
5	29	0.166	1.146	0.829	0.317
6	29	0.267	0.759	0.364	0.395
Total.....		1.270			1.891

conditions of our experiments is 42 per cent of the dry weight of the shoots formed. Hence we must add this value in our final results to a (but not to a_1 , since practically no shoots or roots were formed in the leaves connected with the stems). Table VI gives the dry weight determinations for the six sets of experiments. The dry weight of the shoots produced by all the detached leaves in the six sets of experiments is 1.270 gm. To this value must be added the dry weight of the roots produced which amounts to 42 per cent of the dry weights of shoots; namely, 0.533 gm. The value for a then is $1.270 + 0.533 = 1.803$ gm.

The value for $a_1 + b_1 - b$ was equal to 1.891 gm. Practically no shoots or roots were produced by these leaves connected with their stems, so that no correction for roots is necessary. Hence the value

1.891 represents the gain in mass of the stem. We therefore find that $a = 1.803$, and that $a_1 + b_1 - b = 1.891$. The ratio of $\frac{a}{a_1 + b_1 - b}$ should be $= 1$. In our experiments it is $= 0.95$, which is as near the theoretical value as the accuracy of our experiments permits.

We, therefore, can state: When a leaf is connected with a piece of stem the stem gains in weight and this gain equals the weight of roots and of shoots formed by the sister leaves detached from the stem. The inhibitory effect of the stem upon shoot and root formation by the leaf is therefore adequately explained by these figures as being due to the fact that the material available for growth normally flows from the leaf into the stem.

SUMMARY.

1. Equal masses of sister leaves of *Bryophyllum calycinum* produce equal masses of shoots and roots in equal time and under equal conditions.

2. The mass of shoots and roots produced by different masses of sister leaves in equal time and under equal conditions is approximately in direct proportion to the masses of the leaves.

3. When a piece of stem inhibits the production of shoots and roots in a leaf of *Bryophyllum* connected with it, the stem gains in mass and this gain in mass equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem.

4. This suggests that the inhibitory influence of the stem upon the formation of shoots and roots in the leaf is due to the fact that the material available for this process naturally flows into the stem.

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THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

VOLUME II, NO. 4

MARCH 20, 1920



PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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Entered as second-class matter November 25, 1918, at the Post Office at Baltimore, Md., under the Act of March, 3, 1879

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CORRECTION.

On page 209, Vol. ii, No. 3, January 20, 1920, lines 33 and 36, and page 210, line 20, for $(\text{NH}_4)_2\text{S}$ read NH_4SH .

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A DEVICE FOR REGULATING THE TEMPERATURE OF INCUBATORS EITHER ABOVE OR BELOW ROOM TEMPERATURE.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 23, 1920.)

It is frequently desirable in studying the effect of temperature on living organisms to have a convenient means of regulating the temperature of an incubator or series of incubators within the range of from 5–40°C. For this purpose it is essential to have a regulating device which is reliable over long periods of time with a variation of 0.2–0.3°. As far as the author is aware no convenient method has been described for this purpose.

The device described in this paper has been in use continually for several years and has proved reliable and accurate. It consists essentially in regulating the flow of water through the jacket of a double-walled incubator. This is accomplished, as shown in Fig. 1, by causing a relay to direct a stream of water either through the incubator or to waste as required by the temperature changes. This is brought about by means of a wire D soldered to the armature of the relay and attached at the other end to a glass pipette at the end of a vertical rubber tube. The current necessary to move this wire and pipette is so small that it is unnecessary to use a secondary circuit. The relay is actuated directly by the same circuit which goes through the regulator. With a relay of 150 to 200 ohms resistance, a potential difference of about 1 volt is necessary. This may conveniently be obtained from the ordinary lighting circuit by the use of lamps as shown in Fig. 1. The regulator may be any convenient type, either mercury-toluene or bi-metallic. It is placed in the regular position in the incubator.

The adjustment for temperatures higher than that of the room is as follows: Hot water is allowed to run in a slow stream from the

pipette A which is so adjusted that, when the circuit closes and the armature of the relay is pulled over, the water flows into a funnel B

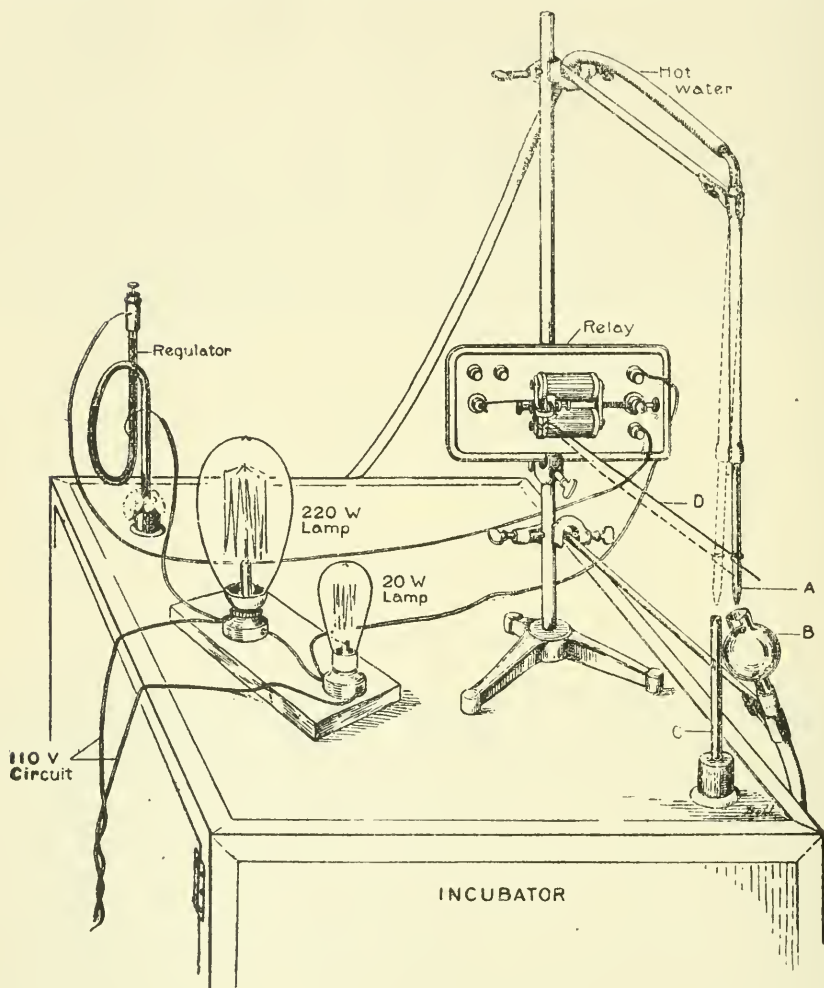


FIG. 1. Device for regulating the temperature of incubators.

placed near the opening C in the incubator jacket and thence to waste. When the circuit is broken the water flows into the incubator and so raises its temperature. The overflow from the incubator

runs off from the top of the water gauge on the side of the incubator (not shown in Fig. 1).

The adjustment for temperatures below room temperature is identical except that cold water is run through A and the relay so adjusted that the water runs into the incubator when the circuit is closed and to waste when the circuit is open. Temperatures to 8 or 10°C. may easily be maintained by the use of ice water.

When temperatures within the range of variation of the room are desired, a slow, continuous stream of cold water is run through the incubator and warm water is run through the pipette connected with the regulating device.

CONCERNING THE HEREDITARY ADAPTATION OF ORGANISMS TO HIGHER TEMPERATURE.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 26, 1920.)

Since the time of Lamarck the theory of the adaptation of organisms to their environment and the inheritance of these adaptations has been a hotly debated question. The original theory considered that the organism responded to changes in environment in such a way as to become better fitted to it, and that these changes then became hereditary. In this form the theory is probably no longer accepted by the majority of students. There is also little doubt that structural adaptations of the individual organisms are not inheritable. A general discussion of this question is out of place here, and the reader is referred to Loeb¹ and Conklin.² It may be pointed out, however, that according to the accepted theory of Weismann in regard to the continuity of the germ plasm, it seems *a priori* improbable that any change in the individual could affect succeeding generations.

Morgan and his coworkers³ have shown that structural changes are continually arising in many forms, and that these changes or mutations are inherited according to Mendel's law. There seems no reason to suppose that physiological changes might not arise in the same way. If these physiological changes were such that the organism became better fitted to a new environment, we might expect to find adaptation under some conditions, although it would not be the direct result of the changed environment. Tower⁴ has reported

¹ Loeb, J., The organism as a whole from a physicochemical viewpoint, New York, 1916.

² Conklin, E. G., Heredity and environment in the development of men, Princeton, 1915.

³ Morgan, T. H., Sturtevant, A. H., Muller, H. J., and Bridges, C. B. The mechanism of Mendelian heredity, New York, 1915.

⁴ Tower, W. L., *Biol. Bull.*, 1917, xxxiii, 229.

experiments which apparently showed some such result. In the bacteria and other unicellular organisms, there is no doubt that cultures may be adapted to very marked changes both in temperature and concentration of toxic substances. This is, however, analogous to the adaptation of an individual multicellular organism, and cannot be considered hereditary in the sense in which the word is used in regard to higher organisms. The individual cells of a bacterial culture bear the same relation to each other as the somatic cells of a single multicellular organism, and are not at all analogous to successive generations of individuals of multicellular organisms.

The experiments reported in this paper were made with aseptic cultures of *Drosophila*. These are especially favorable for such a study for the following reasons. (1) If kept free from microorganisms the results of any experiments made with them become quite regular. (2) They have a very short generation time, about 7 days at 30°. (3) Loeb and Wasteneys⁵ found that the individual *Drosophilæ* show the same marked adaptation to temperature as does *Fundulus*. In the case of the latter, Loeb and Wasteneys found that fish transferred suddenly from 10 to 35°C. died in the course of 1 to 2 hours, whereas fish transferred first to 27° for 2 or 3 days and then put at 35° were able to live indefinitely at this temperature. The results with *Drosophila* showed equally striking individual adaptations.⁶ These experiments were partially repeated and confirmed in the course of the present work.

The relation of the rate of growth and of the duration of life of *Drosophila* to the temperature has been the subject of a previous paper from this laboratory.⁷ It was found in that work that the insects developed normally up to a temperature of 32.5°C. Above this temperature the pupal stage was injured and no further development took place; but the larval and imago stages could live at a temperature several degrees higher. It was also found that increasing the temperature from 10 to 27.5°C. increased the rate of development of the larvæ and pupæ; but that between the temperatures of 27.5 and 32°C. the rate decreased again; *i.e.*, the larvæ grow more slowly at

⁵ Loeb, J., and Wasteneys, H., *J. Exp. Zool.*, 1912, xii, 543.

⁶ Loeb, J., and Wasteneys, H., unpublished data.

⁷ Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103.

either 25 or 30° than they do at 27.5°. This secondary decrease in the rate at temperatures above 27.5° was compared with a similar decrease in the rate of enzyme action, and was ascribed to a similar cause; namely, injury and subsequent slowing up of the growth processes.

In all these experiments the eggs whose development was studied were produced by imagos which had been raised at temperatures of from 15–20°C. If there was any hereditary adaptation to higher temperature it would be expected that flies which had developed near the upper temperature limit would be able to produce eggs slightly

TABLE I.

Effect of Temperature at which Parent Generation Develops on Upper Temperature Limit for Development of the Succeeding Generation.

Temperature at which parent imagos were raised.	Temperature of development of succeeding (F ₁) generation.	Condition of succeeding (F ₁) generation after days noted.			
		2 days.	5 days.	7 days.	15 days.
°C.	°C.				
20	29	Eggs.	Larvæ.	Pupæ.	Imagos.
32	29	"	"	"	"
20	32	"	"	"	"
32	32	"	No larvæ.	No larvæ.	No larvæ.
20	33	"	Larvæ.	Pupæ.	Pupæ dead.
32	33	"	No larvæ.	No larvæ.	No larvæ.

more resistant to temperature (*i.e.* able to develop at a slightly higher temperature) than flies which had developed at a lower temperature. In order to test this assumption, cultures of imagos which had developed at 20 and 32° respectively were placed in incubators at 29, 32, and 33°. The development of the eggs produced by these imagos was then followed. The results are summarized in Table I.

It will be seen that those imagos which had developed at 20° and were then transferred to a temperature of 29 or 32° were able to produce eggs capable of developing into imagos at that temperature. The eggs produced at 33°, however, do not develop beyond the pupal stage. The imagos which had developed at 32° are unable to produce eggs capable of development into imagos at temperatures higher than 29°. The effect of raising *Drosophila* at high temperatures,

therefore, is to lower the upper temperature limit for the development of the succeeding generation and not to elevate it as would be expected if the adaptation to temperature was hereditary.

It might be objected that the results shown in Table I are not due to any difference either in the eggs or imago but merely to the fact that, in the case of the imagos raised at 20°, the eggs which are to give rise to the succeeding generation pass through the early stages of development within the female while at the lower temperature and so escape injury; while in the case of the cultures kept continuously at 32° the early stages of the eggs must necessarily be passed at this temperature and the eggs are thereby injured. If this was the case, only those eggs produced by the 20° culture immediately after being transferred to 32° should develop, and the ones produced later should fail to develop. This, however, is not so. Imagos raised at 20° and transferred to 32° can produce eggs, capable of developing at this temperature, for a week or 10 days after having been transferred from the lower temperature.

It was found that imagos, raised and kept permanently at a temperature of 30°, are unable to produce eggs capable of development at this temperature. If, however, they are removed from the 30° incubator within a week after emerging from the pupæ and placed at a temperature of about 20°C. for 24 hours or longer, they become able to produce eggs capable of development at 30°C. when replaced at this temperature. Table II is a summary of an experiment illustrating this point. It is necessary to remove the imagos from the higher temperature within a week or 10 days after they have emerged from the pupæ. If they are left longer at the higher temperature, the injury becomes permanent and they are no longer able to produce eggs capable of development at any temperature.

It is therefore not possible to raise more than one generation of *Drosophila* at a temperature of 29° or over unless the culture is removed to a lower temperature for at least 24 hours every generation.⁸ A culture has been continued at 30° by this method of intermittent

⁸ This fact may seem surprising since the organism is a tropical form. The explanation is probably that the temperature even in the tropics does not stay continuously higher than 28 or 30° for more than a week or 10 days.

cooling for ten generations without any noticeable change in the upper temperature limit. A second culture was kept continuously at 28° for fifteen generations. In this case also there has been no noticeable change in the temperature limit; *i.e.*, the organisms are still unable to grow for more than one generation at a continuous temperature of 29° or over.

TABLE II.

Effect of Placing Cultures, Raised at 30°, at 22°C.

Days.

1. 50 to 100 imagos from normal aseptic culture generation No. 89 (raised at 20°) placed at 30°C.
2. Many eggs laid.
4. Larvæ developing. Parent imagos removed.
8. Imagos of the new generation (F₁) placed at 22° and put back at 30° after varying time intervals as stated below.

Length of time at 30° C.	Time during which (F ₁) cultures were left at 22°C.				
	0.25 hr.	4 hrs.	24 hrs.	96 hrs.	200 hrs.
Stage of development of (F ₂) generation cultures after days at 30°C.					
days					
3	Eggs, but no larvæ.	Eggs, but no larvæ.	Both eggs and larvæ.	Both eggs and larvæ.	Both eggs and larvæ.
5	Eggs, but no larvæ.	Eggs, but no larvæ.	Both eggs and larvæ.	Both eggs and larvæ.	Both eggs and larvæ.
10	Eggs, but no larvæ.	Eggs, but no larvæ.	Pupæ.	Pupæ.	Pupæ.
15	Eggs, but no larvæ.	Eggs, but no larvæ.	Normal imagos.	Normal imagos.	Normal imagos.

EXPERIMENTAL.

Temperature Control.—The cultures were kept in water jacketed incubators regulated as described in another paper.⁹

Food.—All cultures were fed on a sterilized suspension of yeast in water. The excess water was absorbed by cotton added to the flask, as described below.

Method of Transferring Cultures, Etc.—The insects were kept in 1 liter flat bottom Florence flasks having a side tube fused on as in a Pasteur flask. This side tube was closed with a rubber tube and glass plug, and the neck of the flask plugged with cotton. About 25 cc. of a thick suspension of yeast in water were

⁹ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 309.

added to the flask, the excess water was absorbed by the addition of absorbent cotton, and the flask sterilized. It is important to add sufficient cotton to absorb the water as otherwise the insects stick to the side of the flask and are drowned. In order to transfer the culture, the flask is connected to one containing the insects by means of the side tubes, using the same technique as in handling a Pasteur flask. The flies are then shaken from one flask to the other through the connecting side tubes, the flasks disconnected, and the connecting tubes flamed and re-plugged. In this way the organisms can be handled with as little danger of infection as cultures of bacteria.

SUMMARY.

1. Imagos of *Drosophila* raised at temperatures of from 12–28.5°C. when placed at any temperature from 15–32.5°C. produce eggs which develop normally at these temperatures.

2. Imagos raised at temperatures of from 29–32.5° and then kept permanently within these temperatures produce eggs which do not develop.

3. Imagos raised at from 28.5–32.5°C. and then placed at temperatures of from 12–25°C. produce eggs which develop normally.

4. Imagos raised at from 28.5–32.5°C. placed at 15–25°C. for 24 hours or longer and then put back into a temperature of from 28.5–32.5°C., produce eggs which will develop at the latter temperature.

5. There is no evidence of any hereditary adaptation to higher temperatures.

STEREOTROPISM AS A FUNCTION OF NEUROMUSCULAR ORGANIZATION.

By A. R. MOORE.

(From the Physiological Laboratory of Rutgers College, New Brunswick, N. J., and the Marine Biological Laboratory, Woods Hole, Mass.)

(Received for publication, January 15, 1920.)

When a starfish is put on its back, it rights itself and, though it has been known that this is a case of stereotropism, the details of the reaction have thus far not been accounted for. Thus it has happened that some authors have spoken of this reaction as a case of "trial and error" on the part of the animal. The experiments described in this paper show that the righting reaction of the starfish is a necessary consequence of the neuromuscular organization of the ray and that no room is left for the assumption of "trial and error."

It usually has been assumed by writers on the subject that the movements of the starfish, including those of righting, are accomplished entirely by the tube feet.¹ The writer has observed, however, that nicotinized individuals, in which all the tube feet except a few at the tip of the ray—the "feelers"—are retracted and inactive, begin righting movements, and in some cases complete the righting in course of half an hour or so. This indicates that the essential movements of righting can be accomplished by means of the nerves and muscles of the arms themselves without the use of the tube feet as locomotor organs. It therefore follows that the functioning of the *Asterias* ray in righting is based on principles similar to those which operate in the case of ophiurans. It seems probable that the principal rôle of the tube feet of *Asterias* in normal righting lies in their service as delicate sense organs, making possible quick and accurate functioning of the ray musculature. They also act as accessory locomotor organs and contribute to the speed of the somersault.

¹ Belonius, P., *De Aquatibus*, Paris, 1553.

The Musculature.

The muscles which determine the movement of the ray are classified by position as (a) circular and longitudinal muscles of the dorsal sheath, and (b) interskeletal longitudinal muscles of the ray floor.² The action of the sheath muscles may be demonstrated in the intact animal by stimulating the tip of the ray with a drop of 0.1 N acid. As a result the ray shortens, due to contraction of the longitudinal muscles, and suffers a decrease in diameter from the contraction of the circular ones. Similar results are to be obtained with strong stimulation of the tube feet of any part of the ray, thereby proving the nervous connection of the sensory cells of the tube feet with the muscles of the sheath.

The most complex action of the neuromuscular system is seen in the righting response. Here certain arms bend dorsally, others ventrally, in response to reciprocal impulses.³ Obviously it greatly aids an analysis of such reactions if the investigator can produce them at will artificially or entirely apart from their normal causation. This is made possible in the case of the starfish ray through the use of the alkaloids, strychnine and nicotine.⁴ With strychnine the ray bends dorsally, with nicotine ventrally. The parts played by the various groups of muscles may be shown by separating the dorsal sheath from the floor of the ray by cutting the two structures apart longitudinally. When put into either nicotine or strychnine the dorsal sheath shows dorsal flexure and rolls together so that the cut edges meet and overlap. This demonstrates physiologically the existence of longitudinal and circular muscles in the dorsal sheath. In similar fashion the presence of dorsoflex and ventroflex musculature in the floor of the ray may be shown, since in nicotine the floor bends ventrally, in strychnine dorsally.

There are therefore four muscle groups which play a part in the movements of the ray. (a) The longitudinal dorsoflexors of the sheath; (b) the circular muscles of the sheath, effective in twisting

² Bronn, H. G., *Klassen und Ordnungen des Tier-Reichs wissenschaftlich dargestellt in Wort und Bild*, Leipsic, 1899, ii, 3A, 543.

³ Loeb, J., *Comparative physiology of the brain and comparative psychology*, New York, 1900, 61. Moore, A. R., *Am. J. Physiol.*, 1910-11, xxvii, 207.

⁴ Moore, A. R., *J. Gen. Physiol.*, 1919-20, ii, 201.

movements; (c) the longitudinal interskeletal dorsoflexors of the ray floor; (d) the longitudinal interskeletal ventroflexors of the ray floor.

It will be shown elsewhere that limited parts of each system are independently innervated and are capable of independent action. This permits the ray to bend either dorsally or ventrally at any point, and to twist the peripheral part on its long axis through any angle up to 180° .

Motor Nerve Elements.

It has been found that strychnine⁵ and nicotine are without excitatory action on the neuromuscular system of the cœlenterate *Metridium*. It may be assumed then that the alkaloids in question, in the case of echinoderms and the higher forms, act on elements of the neurone especially developed in the more complex types of nervous system. The contraction of certain groups of muscles as the result of the action of nicotine or of strychnine consequently becomes an indicator of chemical excitation of the corresponding nervous elements. If we assume that the excitatory action of nicotine and strychnine is on some part of the motor neurone such as the sensory-motor junction, it would then follow as to the loci of the action of the alkaloids that (a) both substances excite the motor neurones of the dorsal sheath; (b) both substances excite the motor neurones innervating the muscles of the ray floor which cause ventral flexure; (c) strychnine excites the motor neurones governing the dorsoflex musculature of the ray floor; they are refractory to nicotine.

Sensory Nerve Elements.

Ordinarily when an *Asterias* is laid on its back on the floor of an aquarium the rays begin their dorsal bending quite promptly, and righting is accomplished in 1 or 2 minutes. If, however, the aquarium jar has a cone-shaped floor and the inverted animal is placed so that its central disk rests on the apex of the cone, the righting is much delayed, since in this case the rays only by chance come into contact

⁵ Moore, A. R., *Proc. Nat. Acad. Sc.*, 1917, iii, 598.

with the bottom surface. Or if a specimen of *Asterias* is suspended in the water by a thread tied around the central disk, so that the rays cannot touch a surface, dorsal bending of the rays does not occur at all. The animal hangs inert for an indefinite length of time with the rays in a position of partial ventral flexure (Fig. 1). Now hold a glass rod in contact with the back of the ray. The latter bends slowly a little distance dorsalward (Fig. 2). This proves that the sensory cells of the dorsal integument, when stimulated, cause contraction of the dorsoflexors of the sheath. Ordinarily this reflex plays a part in initiating the righting by pressing the entire ray against the bottom. This enables the sensitive "feelers" of the tip of the ray to touch bottom immediately, and from the numerous sensory cells of the am-

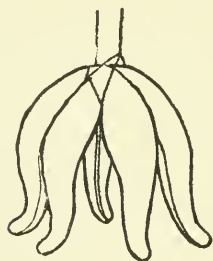


FIG. 1.

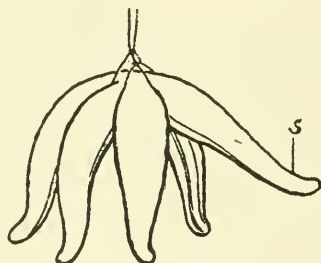


FIG. 2.

FIG. 1. Position taken by an individual *Asterias* during suspension in an aquarium. The animal is not in contact with a surface, consequently the rays are inert.

FIG. 2. A glass rod has been brought into contact with the dorsal side of the ray at the point indicated by *S*. As a result the ray has responded by bending dorsally.

bulacral disks impulses go to the musculature facilitating flexure. As the tube feet secure hold, that part of the ray straightens and actual bending occurs only centralward to the point where the last tube feet have touched bottom.

That the muscular responses of the later phases of righting are due to stimulation of the sensory cells of the tube feet may be shown in the following way. Place a medium sized *Asterias* ventral side up on a glass plate. Stimulate the sensory cells of the tube feet by touching these organs at the tip of the ray with a loop of thread dipped in 0.05 N HCl. At once the tip bends dorsally (Fig. 3). Now touch the tube

feet near the middle of the ray with the acid thread. Two results appear; (a) the tip returns to its former position, and (b) the ray bends dorsally immediately central to the point of the last application of the acid (Fig. 4). Stimulation of the sensory cells of the tube feet, therefore, results in dorsal flexure of the ray central to the point of stimulation, and inhibition distal to this point. It was found as a rule that strychnine abolished this phase of inhibition, so that in a thoroughly strychninized ray stimulation of the tube feet at any point resulted in dorsal flexure of the entire ray.

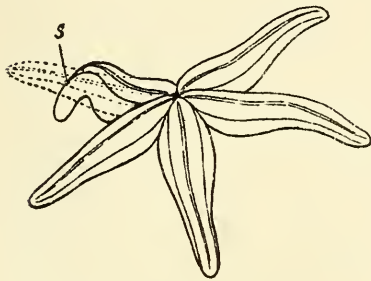


FIG. 3.

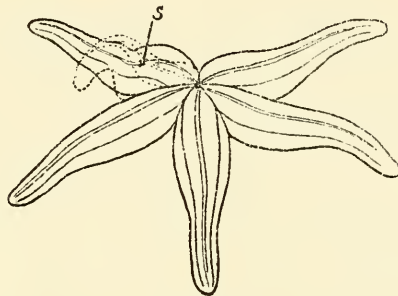


FIG. 4.

FIG. 3. The specimen lies ventral side up on a glass plate. A thread previously dipped in 0.05 N HCl has touched the extended ray (indicated by the dotted outline) near the tip as at S, with the result that the distal end of the ray bends sharply dorsalward.

FIG. 4. With the ray in the position of Fig. 3, shown here by dotted outline, the acid stimulus is applied to the tube feet near the center of the ray. As a result the distal half is extended to the normal position but dorsal flexure occurs central to the point of stimulation.

CONCLUSIONS.

From what has been said concerning the neuromuscular organization of the *Asterias* ray, it is possible to arrive at certain definite conclusions regarding the nature of the stereotropism of that organ. The righting movements from the start are not haphazard; *i.e.*, they cannot be explained on any hypothesis of "trial and error." The excitation of the sensory cells of the dorsal sheath initiates dorsal flexure of the ray. This movement makes it possible for the tube feet of the tip to touch bottom and start the vigorous action which follows and completes the righting. If the "feelers" by chance touch bottom they initiate the reaction *de novo*.

As to the remaining phases, the stereotropic reaction of the ray is referable to the high degree of surface sensitivity of the tube feet disks and the propagation of excitatory and inhibitory impulses, resulting from stimulation, along appropriate paths to the muscles of the ray. In this respect the tube feet may be regarded as playing a rôle similar to that of tropistic receptors in general. That is, unequal stimulation of the receptors causes a corresponding inequality of tone or of contraction in the musculature which ultimately results in an equilibrium of orientation to the factor involved.

It is evident that as a mechanism for righting stereotropism differs in one important respect from other tropisms. The latter depend for their operation upon unilateral effects in organisms which are dynamically bilaterally symmetrical. In stereotropism the sensitivity of the organism is not distributed in bilaterally symmetrical fashion. As a rule only the ventral side is stereosensitive; *i.e.*, the sensitivity is unilaterally distributed. There is a tropistic action in such cases because of the fact that when the sensitive surface is only partially stimulated an unequal contraction of the musculature follows and this as a result brings the sensitive surface into such a position that it is all equally stimulated. Equal muscle tone follows and the organism is in equilibrium with its environment.⁶

⁶ Jennings (Jennings, H. S., *Univ. California Pub., Zoology*, 1907-08, iv, 156) believes that he has discovered in *Asterias* a capacity for learning in connection with this reaction; that is, a ray ordinarily passive during righting could be induced by repeated suppression of the other more active rays to undertake righting movements. In describing his experiments Jennings states that the active rays were prevented from taking part in the righting by "stimulating their tube feet with a glass rod." In 1910 (Moore, A. R., *Biol. Bull.* 1910, xix, 235), I called attention to the fact that Jennings' result did not prove the use of memory or hysteresis of any kind in this functioning of a previously passive ray, because strong stimulation or a slight injury of any sort to the other rays renders them inactive and forces the use of the ray in question. By "stimulating with a glass rod," Jennings simply diminished the reacting capacity of the active rays, or, in other words, raised their threshold either to a point equal to or above that of the ray he was seeking to "teach." The experiment may be much simplified by making a single stroke with a glass rod in the ambulacral groove of each of the four active rays. Animals treated in this way have been kept under observation and found to resume the use of the treated rays only after 1 or 2 weeks. In the interval righting is always performed by the previously inactive ray which, however, had not been stroked along the groove with a glass rod.

REGENERATION AND NEOTENY.

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(Received for publication, January 29, 1920.)

According to Kammerer, it is possible to prevent metamorphosis in the caudate amphibians by amputation of the legs or tails of the larvæ. In my attempts to produce neoteny in the larvæ of salamanders, however, Kammerer's method has been tried without success, my experiments failing completely to confirm Kammerer's positive statements with regard to the effectiveness of the method.

Dumeril¹ observed that among several larvæ of *Ambystoma tigrinum*, which were the offspring of neotenuous animals (axolotls), only those metamorphosed which had been deprived by their comrades of legs and part of their tails. He believed, therefore, in contradistinction to Kammerer, that regeneration may induce metamorphosis in larvæ which without regeneration would become neotenuous. Later, however, he observed that larvæ of the same lot, which had not been mutilated, also metamorphosed; hence he was finally convinced that regeneration had nothing to do with metamorphosis of the larvæ.

Kammerer² experimented not only on the larvæ of caudate amphibians (*Triton cristatus* and *Triton alpestris*), but also on those of *Salientia*. He amputated the limbs as well as the tail. He concluded from his experiments that

"Injuries of any sort effect metamorphosis in directly opposite ways in Urodela and Anura as demonstrated with greatest certainty in the experiments. While in the salamander larvæ neoteny is brought about without the slightest difficulty, if only one limb or a piece of the tail is removed, the same procedure induces a rapid appearance of the transformation symptoms in the tadpole."³

¹ Dumeril, A., *Ann. Sc. Nat. Zool.*, 1867, vii, 229.

² Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1905, xix, 148.

³ Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1905, xix, 176.

Though such a difference between two different groups of animals is possible, it does not seem likely that metamorphosis should be controlled in different groups of amphibians by mechanisms so different; at least such an assumption does not seem justified by the evidence presented in Kammerer's paper. In the first place, that which Kammerer calls neoteny in his *Triton* larvæ is, as far as his recorded data are concerned, only a difference of 1 month. In one instance the operated animals transformed 1 month later than the controls; in the second experiment the operated larvæ had not metamorphosed 14 days after the controls had completed metamorphosis; at this time the experiments were discontinued.⁴ Nowhere do we find any indication that the sex organs were actually examined to make sure that they had developed at a more rapid rate than the rest of the organism. Nor do we find any proof that these small differences had not been produced merely by differences in the quantity of food or that they were not due to the fact that the larvæ of the different sets were the offspring of different females. Since the same objections could be raised with regard to his experiments on tadpoles, it becomes doubtful not only that so fundamental a difference exists between *Caudata* and *Salientia* as that claimed by him, but also whether amputation and regeneration had any effect on the metamorphosis of Kammerer's larvæ at all. In a later article⁵ he emphasized the fact that the retardation of metamorphosis in his larvæ was not due to a retardation of growth because of insufficient food; he says:

"Individuals particularly suited for the production of the phenomena of neoteny are those which have been subjected to experiments on regeneration, since they as a rule retain . . . for a long time after the removed parts have been replaced the larval condition without showing any particular inhibition of the general growth of the body; hence they turn into truly neotenuous, not into starved larvæ!"⁶

It is in this case of course extremely difficult to form any opinion about the causes which lead to retarded metamorphosis, since apparently these animals were well fed, but it is well known, and we have

⁴ Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1904, xvii, Experiment XI, p. 167, and Experiment XII, p. 168.

⁵ Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1904, xvii, 165.

⁶ Kammerer, P., *Arch. Entwicklungsmechn. Organ.*, 1904, xvii, 240.

discussed it in a previous article,⁷ that the larvæ of all species so far examined must grow longer in low temperature than in high temperature before metamorphosis can take place, without, however, under these circumstances becoming neotenous. Unfortunately Kammerer does not mention at what temperatures his larvæ were kept. In the same article Kammerer refers to neotenous larvæ of *Salamandra maculosa*, 2 years old; it is very probable that these larvæ were truly neotenous. The experiments reported in this paper make it, however, very doubtful that this result could have been obtained by merely cutting off the limbs and the tails. I tested the effect of the removal of the limbs and of the tail followed by regeneration of these parts first in the species *Ambystoma opacum*. In the fall of 1916 the eggs of one female were collected and twenty-eight of them divided into four series; two series (E and G), consisting of six larvæ each, were used as experimental series, E being kept at approximately 25°C., G at approximately 15°C.; each experimental series was controlled by a series consisting of eight animals (A and C). The larvæ were measured and examined at least once every week. They were kept in individual jars, and individual records were made. Both fore limbs were removed from the larvæ of Series E and G 46 days after hatching, and 88 days after hatching 50 per cent of the tail was cut off. In Series E the regeneration of the legs was nearly completed 102 days after hatching, *i.e.* 80 days before metamorphosis; in Series G the legs had not regenerated to their normal length 109 days after hatching, but from this time on regeneration occurred at a very slow rate, and the animals never possessed legs of normal length. Regeneration of the tails was most vigorous during the first weeks after they had been cut off but continued in both series throughout the larval period.

Table I shows the result of this experiment. For reasons discussed in other papers,⁷ metamorphosis was regarded as taking place at the time when the first molt occurred; consequently the figures recorded in this table represent the number of days after which the animals shed their skins for the first time. In several larvæ this figure was not recorded, the date when they were set on land being recorded instead. At 25°C. this was done on the same day as the first molt, or 1 day

⁷ Uhlenhuth, E., *J. Gen. Physiol.*, 1918-19, i, 525.

later, and the error caused by this difference is very small. At 15°C. in only one instance was the day of the first molting not recorded, and the error in this instance may amount to from 1 to 4 days. As the figures show, there was no difference between the controls and the experimentals at 15°C., both series metamorphosing 243 days

TABLE I.

Regeneration and Metamorphosis in Ambystoma opacum.

All four series fed on earthworms; A and E kept at approximately 25°C. average temperature, C and G approximately 15°C. average temperature. In E and G, at 46 days after hatching, both fore limbs were cut off, and at 88 days 50 per cent of the tails was cut off.

No.	25°C.		15°C.	
	A Control.	E Regenerating.	C Control.	G Regenerating.
	days	days	days	days
1	191(Land.)	216(Land.)		236
2	200	206	241	235
3	169(Land.)	182(Land.)	249	+
4	184	+	234	239
5	186	209(Land.)	247	+
6	+	199	+	261(Island.)
7	172		242	
8	199		+	
Average.....	186	202	243	243
Difference.....		16		0

after hatching. At 25°C. the difference was very small; the regenerating series metamorphosed only 16 days later than the controls.

These results show that in *Ambystoma opacum* neoteny cannot be produced by removal of parts of the body and their regeneration. Beyond this no conclusions can be safely drawn. It should be mentioned, however, that in all four series approximately the same amount of food was available for the animals. The earthworms which served as food were given in pieces of approximately the same size. Since, however, the amount of food in these series was not large enough to cover the demand of normal growth, the animals were partly starved, as may be seen also from the length of the larval period, which amounts

to 186 days in Series A, while it is only 60 to 100 days at 25°C. if an unlimited supply of food is allowed the larvæ. For this reason it is possible that at high temperature (25°C.), at which regeneration proceeds at a more rapid rate than in lower temperatures, the regenerating larvæ were less well supplied with food than the controls, notwithstanding that both received an equal amount, and that this circum-

TABLE II.

Regeneration and Metamorphosis in Ambystoma tigrinum.

Both series kept at 15°C. In Series LVI the fore legs were removed at 26 days, from 4.5 to 11 cm. of the tails were removed at 47, 61, 68, 82, 96, 110, 124, and 138 days, and the hind legs were removed at 61 days.

No.	XLVIII Control.	LVI Regenerating.
	<i>days</i>	<i>days</i>
1	123	124
2	131	145
3	145	148
4	122	130
5	120	148
6	137	122
Average.....	130	136

stance caused the delay in metamorphosis in Series E. The experiments were apparently unsatisfactory also because they did not warrant a generalization of the conclusion that neoteny could not be brought about by removal of the parts of the body, since the species used might be less prone to neoteny than *Triton* and *Salamandra maculosa*.

Accordingly the experiment was repeated in the spring of 1919 with the larvæ of *Ambystoma tigrinum*, a species frequently found in neotenuous condition. With respect to food, a more satisfactory condition was established by placing in the jars every day an amount of earthworms greater than was required by the larvæ. Two series, a regenerating (LVI) and a control (XLVIII), were kept at 15°C.; both were the offspring of the same female, and each consisted of six animals. They were kept in separate jars and the observations

recorded individually. In Series LVI the fore limbs were removed at 26 days, the hind limbs at 61 days, and 11 cm. of the tail at 47 days after hatching. To assure continuous regeneration the tails were clipped as soon as part of the previously removed tips had regenerated (at 47, 61, 68, 82, 96, 110, and 124 days after hatching).

The result is summarized in Table II. It was practically the same as that of the first experiment. The larvæ of Series LVI metamorphosed (*i.e.* shed the skin for the first time) 6 days later than the controls. Since this difference is smaller than the differences observed among the larvæ of the same series it may be said that in both series the larvæ metamorphosed at the same time. And certainly there was no neoteny produced by removal of even considerable amounts of tissue (42.4 mm. of tail were removed by the successive clippings of the whole larval period), though the species used is one which would be expected to yield readily to influences producing neoteny in a species like *Salamandra maculosa*, which only rarely is found in neotenuous condition.

CONCLUSIONS.

It is apparently quite certain that removal of parts of the body (limbs, tail) followed by regeneration of these parts (1) does not produce neoteny in the larvæ of salamanders, and (2) has no influence upon metamorphosis.

COMPARATIVE STUDIES ON RESPIRATION.

X. TOXIC AND ANTAGONISTIC EFFECTS OF MAGNESIUM IN RELATION TO THE RESPIRATION OF *BACILLUS SUBTILIS*.

By MATILDA MOLDENHAUER BROOKS.

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(Received for publication, January 14, 1920.)

The behavior of magnesium is of special interest since in studies on antagonism magnesium has been found to occupy an intermediate position between the monovalent and the bivalent metals. In many cases it can act as an antagonist to calcium as well as to sodium. As no investigations on respiration have been undertaken from this point of view, the writer has made some experiments on this subject.

The experiments of Lipman¹ on the rate of ammonification of *Bacillus subtilis* have shown that there is some antagonism between sodium and magnesium. On the other hand he found no antagonism, but increased toxicity, when magnesium and calcium were combined.² Kelley,³ in studying the ammonification and nitrification of certain soils, found no antagonism between magnesium and sodium. This sort of antagonism was observed, however, in the case of nitrogen fixation by *Azotobacter chroococcum*, as reported by Lipman and Burgess.⁴

In view of these facts it seemed desirable to study the effects of magnesium upon the respiration of *Bacillus subtilis*. The technique employed was the same as that described in a previous publication.⁵

¹ Lipman, C. B., *Bot. Gaz.*, 1910, xlix, 207.

² Lipman, C. B., *Bot. Gaz.*, 1909, xlviii, 105.

³ Kelley, W. P., *Univ. of California Pub., Agric. Sc.*, 1912, i, 39.

⁴ Lipman, C. B., and Burgess, P. S., *J. Agric. Sc.*, 1914, vi, 484.

⁵ Brooks, M. M., *J. Gen. Physiol.*, 1919-20, ii, 5.

Rate of CO₂ production

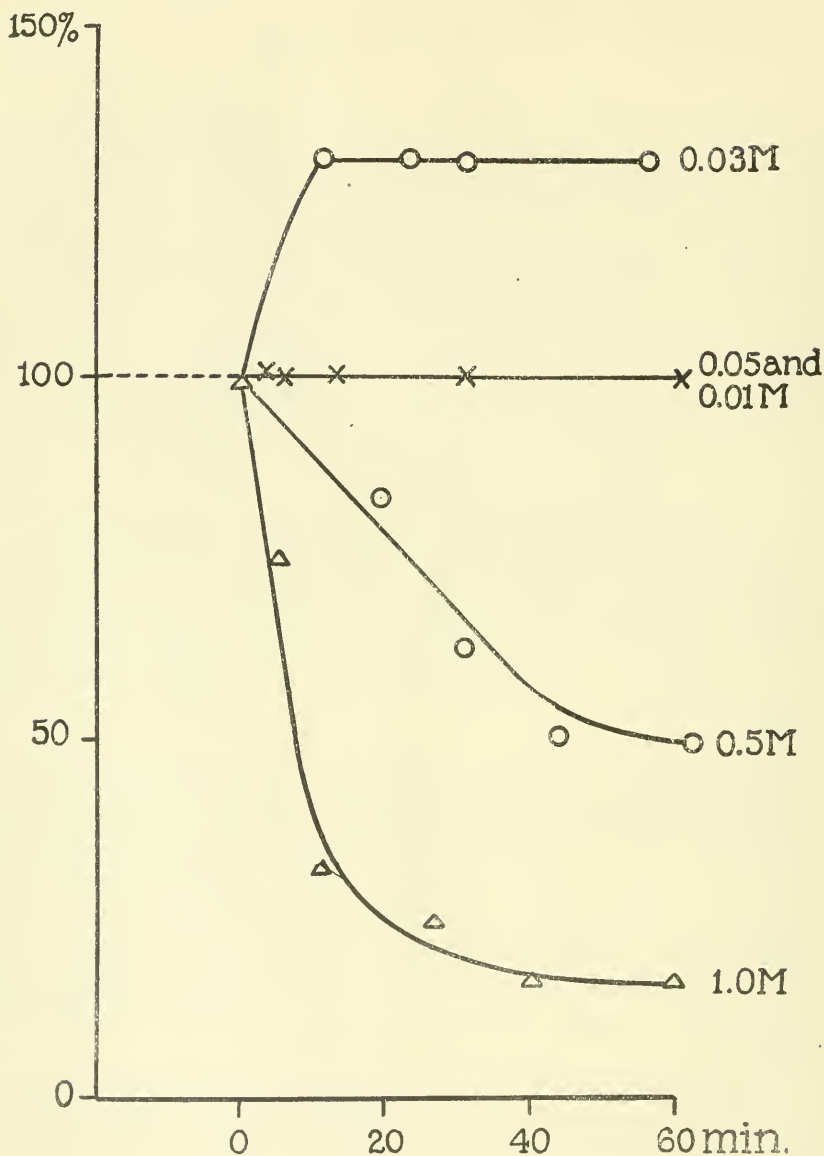


FIG. 1. Curves showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) in 0.03, 0.05, 0.01, 0.5, and 1.0 M MgCl₂. The zero point on the abscissa denotes the beginning of exposure to the salt solution; previous to this the bacteria were in 0.75 per cent solution of dextrose in distilled water. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Each curve represents a single typical experiment.

Fig. 1 shows the manner in which the rate of production of CO_2 changes under the influence of MgCl_2 , in concentrations of 0.01, 0.05, 0.03, 0.5, and 1.0 M. During the first 10 minutes the bacteria are under normal conditions and the curve (broken line) is horizontal. After this, at the point marked 0 on the abscissa, the salt is added. For example, the addition of sufficient MgCl_2 to make the concentration 0.03 M produces an increase in the rate which remains constant during the period of experimentation. When the concentration is 0.05 and 0.01 M the rate is normal, while in higher concentrations there is a decrease in rate. These curves are selected from a number of similar typical curves and each represents one experiment.

Fig. 2 shows the effect of various concentrations of MgCl_2 upon the rate of respiration expressed as per cent of the normal. The rate indicated is that produced after the bacteria had been in contact with the salt for 1 hour. The figure shows that there is an increase in the rate of production of CO_2 at 0.03 M and a decrease in rate at concentrations higher than 0.05 M.

The effect of osmotic pressure in these experiments is probably negligible, since it has been shown in a previous paper⁵ that respiration is normal in a properly balanced solution obtained by mixing 1.0 M KCl and 1.0 M CaCl_2 in the proper proportions.

Fig. 3 shows the antagonism between the salts. Thus Curve A shows that when 9.3 parts of 0.8 M NaCl, and 0.7 parts of 0.8 M MgCl_2 , were added to the bacteria, the rate of respiration remained normal. The other proportions did not give normal rates.

Curve B illustrates the effect of combinations of CaCl_2 and MgCl_2 upon the rate of production of CO_2 . There is no normal rate with any combination of these salts at 0.8 M concentration. There is a maximum in the curve at 8 parts of 0.8 M CaCl_2 , combined with 2 parts of 0.8 M MgCl_2 , where the antagonism is slight. In experimenting upon lower concentrations of Ca and Mg in the same proportions, no normal rate was obtained until the concentrations were so dilute that they would of necessity, according to their separate curves, produce normal rates. It may be added that the combination of the two monovalent salts, NaCl and KCl, does not give a normal rate.

In order to find out whether the pH value of the liquid containing the bacteria was changed when the salts were added, thereby influ-

Rate of CO₂ production

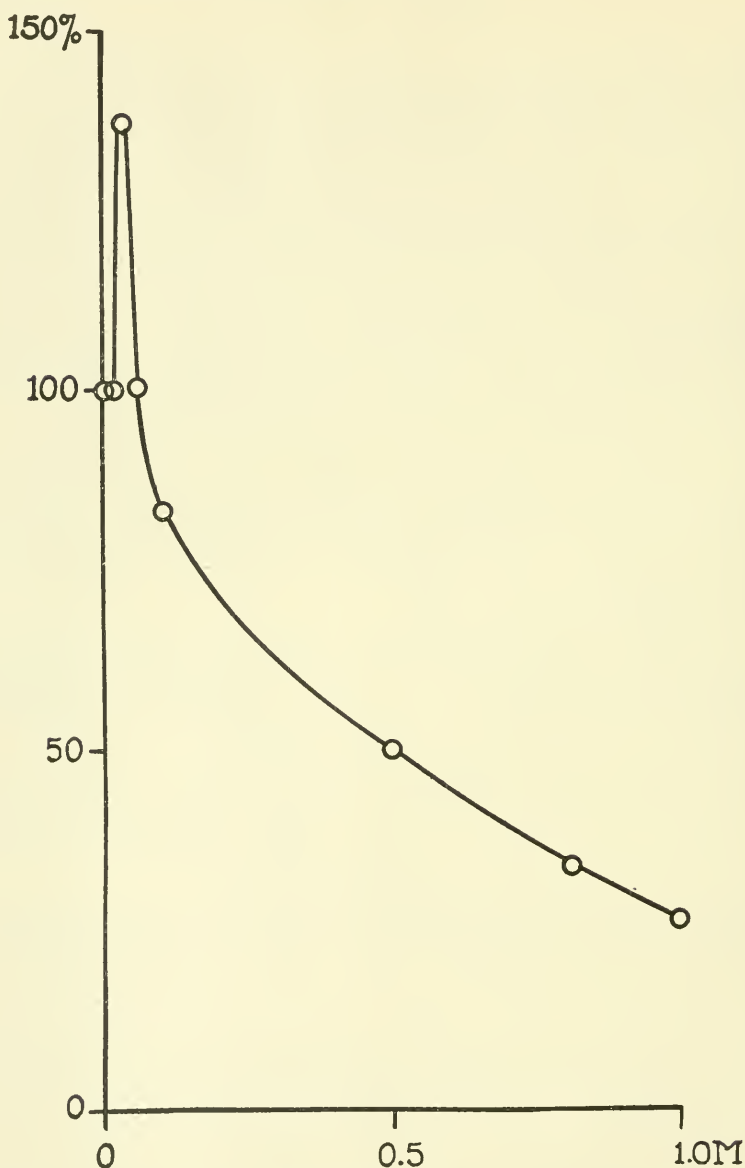


FIG. 2. Curve showing the rate of respiration of *Bacillus subtilis* (expressed as per cent of the normal) as affected by various concentrations of MgCl₂. The normal rate (which is taken as 100 per cent) represents a change in pH value from 7.78 to 7.60 in a number of seconds depending upon the amount of bacterial suspension used, usually 30 seconds. Average of three experiments; probable error less than 3 per cent of the mean.

Rate of CO_2 production

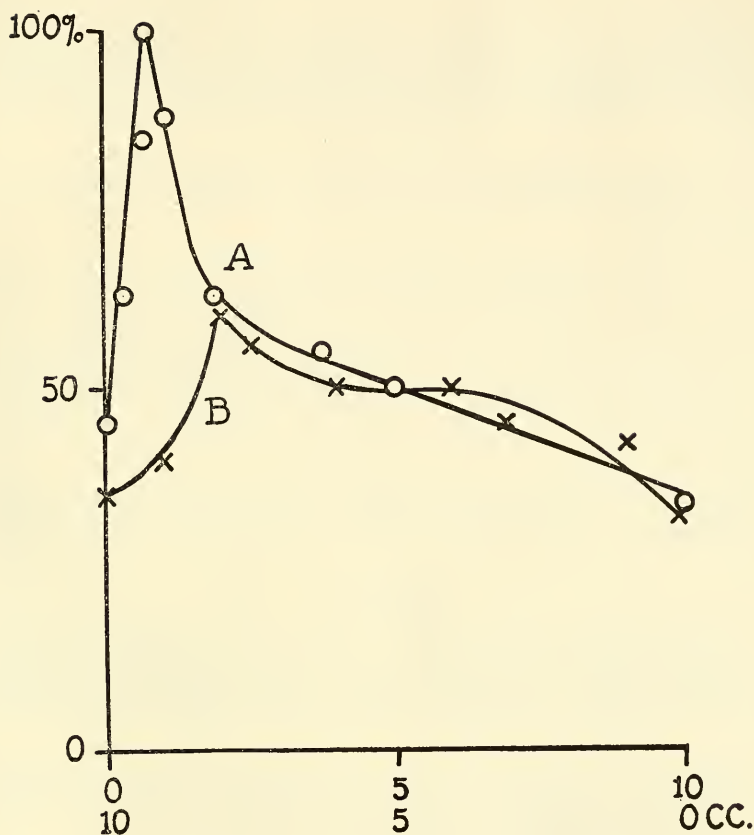


FIG. 3. Curves showing antagonism in the effect of salts on the respiration⁸ of *Bacillus subtilis* (expressed as per cent of the normal). Curve A, antagonism between 0.8 M NaCl (left), and 0.8 M MgCl_2 (right); Curve B, antagonism between 0.8 M CaCl_2 (left) and 0.8 M MgCl_2 (right). The ordinates represent rate of respiration (expressed as per cent of the normal); the abscissæ represent molecular proportions of the salts used. Thus, in Curve A, the ordinate at the extreme left represents the rate in 0.8 M NaCl, while the ordinate at the extreme right represents the rate in 0.8 M MgCl_2 . The ordinate in the middle represents the rate in 50 parts of 0.8 M NaCl, and 50 parts of 0.8 M MgCl_2 . In Curve B the ordinate at the extreme left represents the rate in 0.8 M CaCl_2 , while the ordinate at the extreme right represents the rate in 0.8 M MgCl_2 . The normal rate (which is taken as 100 per cent) represents a change in pH from 7.78 to 7.60 in about 30 seconds, varying according to the number of bacteria used. Curve A, average of three experiments; Curve B, average of three experiments. Probable error less than 3 per cent of the mean.

encing the rate of respiration, an indicator was added to this liquid in the apparatus; the pH value was observed to remain so nearly constant that the change in the rate of respiration could not be attributed to changes in alkalinity of the medium in which the bacteria were placed.

The control experiments gave the same results as described in a previous paper.⁵

The results are in accord with those of Lipman, except that they show a slight antagonism between magnesium and calcium. The difference between these observations and those of Lipman may be due to the differences in concentration, or in the length of the experiments, or to other matters of technique. It is possible that a difference in the strain of bacteria may have something to do with the result.

In general the results are in accord with those obtained in studies on antagonism in which criteria other than respiration are employed.

SUMMARY.

1. Concentrations of MgCl_2 up to 0.01 M have little effect upon the rate of respiration of *Bacillus subtilis*; at 0.03 M there is an increase in the rate, while in the higher concentrations there is a gradual decrease.

2. There is a well marked antagonism between MgCl_2 and NaCl , and a very slight antagonism between MgCl_2 and CaCl_2 .

INTENSITY AND THE PROCESS OF PHOTORECEPTION.

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(Received for publication, January 22, 1920.)

I.

Photoreception in animals like *Mya* and *Ciona* is essentially a two-fold process. This is evidenced by the duality of the reaction time in its division into an initial exposure period and a subsequent latent period. The underlying mechanism of photoreception follows this composition of the reaction time. During the exposure or sensitization period a photochemical reaction is initiated in which a photosensitive substance *S* is decomposed into its precursors *P* and *A*. In the subsequent latent period these freshly formed precursors serve to catalyze an independent reaction involving the transformation of an indifferent material *L* into an active substance *T*. This active material *T* sets off the nervous impulse for a response (Hecht, 1918-19, *a*, *b*).

The exact interrelation between the two reactions of photoreception and the external energy is of importance in a complete understanding of the nature of the receptor mechanism. Of the two processes only the initial photochemical reaction is amenable to direct experimental modification. However, the duration of the subsequent latent period reaction is dependent on the products of the photochemical reaction. In addition, variations in the velocity of the latent period reaction are easily measureable with a stop-watch. These circumstances enable one to study quantitatively the effect of changes in the photochemical reaction on the properties of the latent period reaction.

The amount of fresh precursors produced by the initial photochemical reaction is a function of the available energy. Both components of this energy can be controlled independently, and their actions investigated. The effect of changes in the duration of exposure at con-

stant intensity has already been studied. The results show that within the investigated range of exposure the velocity of the latent period reaction is a linear function of the exposure time (Hecht, 1918-19, *b*). It is assumed that the velocity of the latent period reaction is directly proportional to the concentration of the catalytic substances *P* and *A*. Therefore the photochemical effect of the light is a linear function of its time component.

The limits between which the *time* factor may be varied in these experiments are only a few hundredths of a second apart. This is due to the short exposure which is required for a response (Hecht, 1919-20, *c*). The *intensity* factor, however, may be varied over a much greater range, and with more precision even than the time factor. The relation between the source of energy and the primary and secondary reactions may therefore be determined for a wider gradation of energy application than has been done heretofore.

With this in mind, the present series of experiments was performed. The animal used is *Mya arenaria*. For a description of the sensory properties of this animal the reader is referred to previous papers of this series (Hecht, 1919-20, *c*). The work was done at the Marine Biological Laboratory at Woods Hole, Massachusetts, during the summer of 1919.

II.

The experiments are very simple. A number of animals are thoroughly dark-adapted by being kept in a dark room for 24 hours. Using a constant source of illumination and a constant exposure time, the reaction time of each animal is determined at different distances from the light. After each observation, the animal is maintained in complete darkness for 15 minutes before the next observation is made on it. As the duration of the exposure to light is known, the latent period is found by subtracting the exposure from the reaction time. Also, since the source of illumination is a concentrated-filament, incandescent lamp, the intensities may be computed from the distances on the inverse square law. The exposure used was 0.133 second, and the source of light a 250 watt Mazda lamp. The arrangement and construction of the apparatus will be found in detail in a recent publication (Hecht, 1919-20, *c*).

Two sets of experiments were made, the first with five animals and the second with four animals. The results were identical in both series. Indeed the individual animals gave essentially the same re-

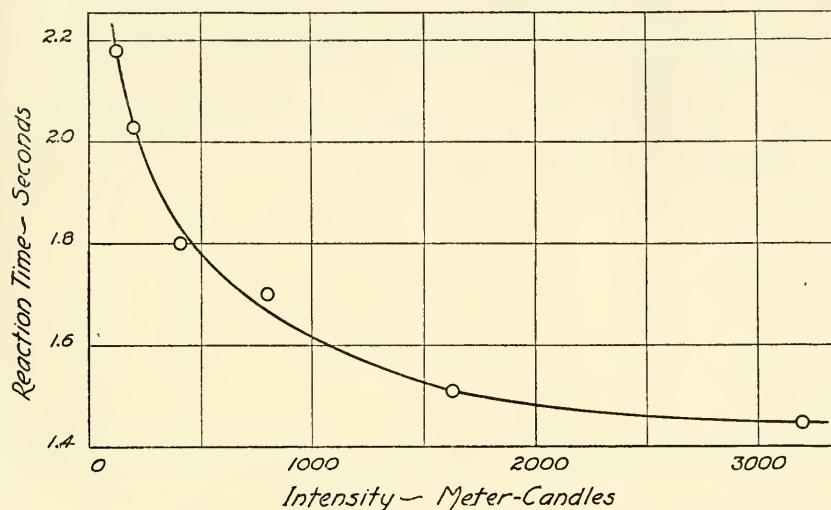


FIG. 1. Data showing the effect of variations in intensity at constant exposure on the duration of the reaction time of *Mya*. Each point is the average of fifteen readings, three each on five animals.

TABLE I.

Relation between the Latent Period and the Intensity at Constant Exposure.

Experiments 121 to 125 inclusive; exposure, 0.133 second.

Intensity.	Reaction time.	Latent period.
<i>meter candles</i>	<i>sec.</i>	<i>sec.</i>
118	2.18	2.05
200	2.03	1.90
408	1.80	1.67
800	1.70	1.57
1,630	1.51	1.38
3,200	1.45	1.32

sults as the averages for either set of experiments. Fig. 1 and Table I give the data for the first set of five animals. Each reaction time in the table is the average of fifteen observations, three on each of the

five animals. From Fig. 1 it is clear that the duration of the latent period varies inversely with the intensity of the stimulating light.

This conclusion is precisely what is to be expected from previous work. It indicates definitely enough that the velocity of the latent period reaction is controlled by the products of decomposition of the photochemical reaction. The data are, however, not to be dismissed with a merely qualitative treatment. A closer analysis brings to light a situation of the utmost significance for an understanding of the mechanism of photoreception.

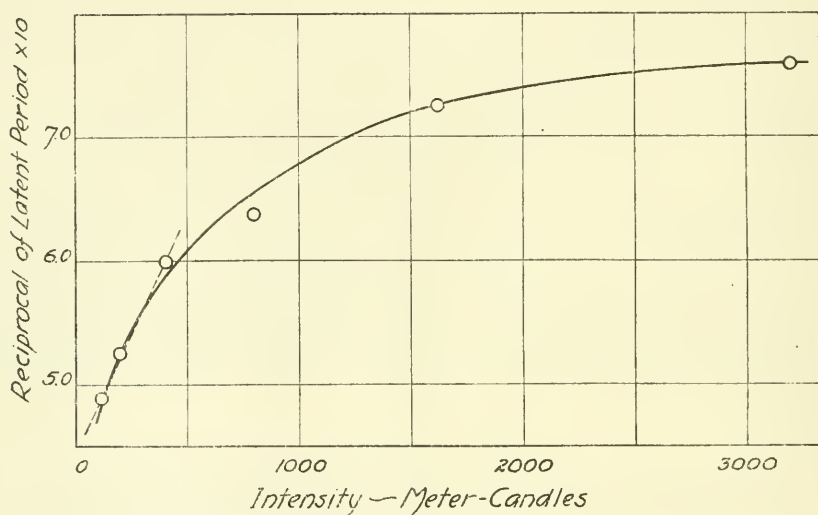


FIG. 2. Relation between the intensity at constant exposure and the velocity of the latent period reaction. The velocity as given by the ordinates is ten times the reciprocal of the latent period.

III.

The reciprocal of the latent period is a direct measure of the velocity of the reaction $L \rightarrow T$ which determines the duration of the latent period. Fig. 2 gives the connection between the intensity and the velocity of the latent period reaction. For convenience the velocity is represented as ten times the reciprocal of the latent period. This makes no difference in the theoretical deductions, because it merely changes the units in which the velocity is given. From the figure it

is at once apparent that the relation between the velocity and the intensity is not linear. What the relation is does not appear definitely, but the smoothed curve passing through the points is decidedly logarithmic in appearance. This is substantiated by Fig. 3 in which the logarithm of the intensity is used as abscissa rather than the intensity itself. The fact that the curve becomes a straight line shows that the velocity is some logarithmic function of the intensity.

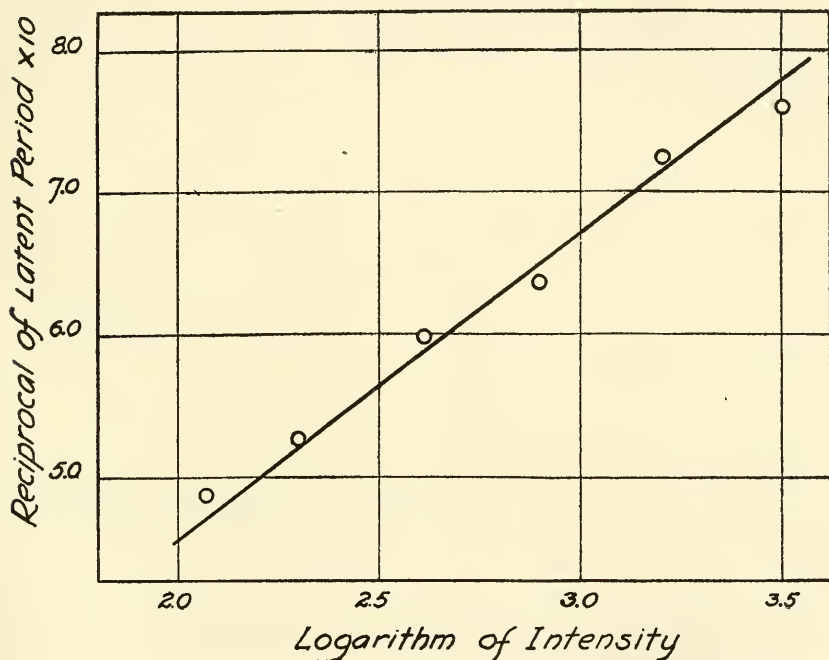


FIG. 3. Relation between the velocity of the latent period and the logarithm of the stimulating intensity. The points are the same as in Figs. 1 and 2. The straight line is drawn passing through the center of coordinates and has a slope of 2.2.

To find the exact correspondence between the two, a simple mathematical treatment is sufficient. The equation of a straight line is

$$y = ax + b \quad (1)$$

in which a is the slope of the line, x and y the abscissa and ordinate respectively, and b is the distance above the center of coordinates at

which the line crosses the y axis. The line in Fig. 3 crosses the coordinates at $(0, 0)$. This is true graphically, and also follows from the fact that at zero intensity the velocity of the latent period reaction is zero. Therefore $b = 0$, and the equation becomes

$$y = ax. \quad (2)$$

Using the notation of Fig. 3, y is the velocity V , and x is the logarithm of the intensity. The numerical value of the constant a is found by dividing a given value of the ordinate by the corresponding value of the abscissa. In Fig. 3, $a = 2.2$. Equation (2) may therefore be written

$$V = 2.2 \log I. \quad (3)$$

It will be remembered that we have been using common logarithms. The factor for converting common into natural logarithms is 2.3. It is highly probable that within experimental errors, our constant $a = 2.2$ is the same thing as the factor for converting Briggsian into Napierian logarithms. The equation for the straight line in Fig. 3 should therefore be

$$V = \ln I \quad (4)$$

in which \ln means logarithms to the base e .

Equation (4) not only demonstrates the logarithmic connection between the incident light and the velocity of the latent period, but it shows that this relation is of the simplest mathematical nature. Before making any theoretical deductions from equation (4), it will clarify matters if we first consider its direct connection with the reactions which underlie photic sensitivity.

IV.

The two terms of the equation which we have just deduced represent the initial step and the final result of the double process of light sensitivity. The light decomposes a photosensitive substance into its precursors. These precursors, according to our hypothesis, then catalyze the latent period reaction, the end-product of which initiates the nervous impulse. We have discovered a simple mathematical relation between the intensity of the light and the velocity of the

latent period. The physical connection between these two is, however, not direct. It is made by the mediation of the freshly formed precursor substances. Strictly speaking then, equation (4) is subject to two interpretations. It may represent either the photochemical effect of the light or the catalytic effect of the precursors.

Actually, however, the latter interpretation is excluded. We have assumed that the relation between the concentration of precursors and the velocity of the latent period reaction is linear. "Such proportionality between concentration of the catalyst and the velocity of reaction is found to hold in numerous enzyme reactions within quite wide limits of concentration" (Euler, 1912, p. 132). Equation (4) because of its logarithmic nature cannot therefore represent the catalytic effect of the freshly formed precursors on the reaction of the latent period. It must consequently express the photochemical action of the light and should then be written

$$E = \ln I. \quad (5)$$

Here E means photochemical effect as measured by the decomposition of the photosensitive substance S into its precursors P and A . If it were possible E would be written in grams of precursors formed by the light. As it is, it must be expressed in terms of the velocity of the latent period reaction, which is directly proportional to the concentration of precursor substances.

V.

Equation (5) as it stands is simple and clearly expresses the facts as we found them experimentally. The intensity I is the independent variable and the photochemical effect E is the dependent variable. The facts may, however, be stated in the reverse manner by saying that the intensity is an exponential function of its photochemical effect. Equation (5) then becomes

$$I = e^E \quad (6)$$

all the terms possessing their previous significance.

The differential of the last equation (6) states that

$$\frac{dI}{dE} = kI \quad (7)$$

which means that the increase in intensity necessary to produce an infinitely small increase in photochemical effect is directly proportioned to the intensity itself. Let the intensity I_0 produce the photochemical effect E_0 , and the intensity I_1 produce the photochemical effect E_1 . Then if

$$E_1 - E_0 = E \quad (8)$$

equations (6) and (7) tell us that

$$I_1 = I_0 e^E \quad (9)$$

and that

$$k = \frac{1}{E} \log \frac{I_1}{I_0} \quad (10)$$

k being a constant. In our data $k = 0.43$, which is the factor for converting natural into common logarithms used in equation (10). If natural logarithms are used, k becomes unity as we have previously found.

The significance of equations (5), (6), (7), (9), and (10), particularly of the latter two, is quite apparent. They are all different mathematical forms of the law expressing the variation of a function at a rate proportional to itself. This is a fundamental principle, which Lord Kelvin has called the "compound interest law in nature," and forms the basis of such regularities as Wilhelmy's law for the velocity of chemical reactions, and Newton's law of cooling. For our immediate interest it is significant that this very principle applies to the absorption of light passing through an absorbing medium (Lambert's law, and Beer's law).

Because of the basic similarity between the expressions for the absorption of light and for the photochemical action of light in photoreception it may possibly be that our results depend upon some constant absorbing medium in the sense organ. In that event the photochemical effect *per se* would be directly proportional to the energy transmitted by this absorbing layer to the photosensitive substance behind it.

Although a greenish black pigment is found scattered over the photosensitive siphon of *Mya* it is hardly likely that this acts as such an absorbing medium. The pigment is distributed thinly and irregu-

larly over the surface, and its maximum concentration is near the tip *within* the siphon, where the light reaches it only after it has passed through the sensitive surface. Moreover, individuals vary widely in the amount of pigment they display, some being practically free from it.

Whether our results are ultimately due to the property of an absorbing medium in the sense organ, or whether the equation represents a basic photochemical phenomenon cannot therefore be decided at present. Certain it is that some purely photochemical effects present a similar condition. For example during the period of normal exposure of a photographic plate, the photochemical effect is a logarithmic function of the intensity (Weigert, 1911, p. 86). The final meaning of our results will therefore await the elucidation of similar data in photochemistry proper.

It may be pointed out that the logarithmic relation between the intensity of the light and its effect in photoreception agrees with the general idea expressed in the Weber-Fechner law. This agreement, however, is more apparent than fundamental. The Weber-Fechner law is itself merely a psychophysiological statement of the general "compound interest" principle which our results also follow. The comparison does bring out the fact that a logarithmic relation is not peculiarly a biological phenomenon, as much of the discussion of the Weber-Fechner law may lead one to suppose. It is well known in physical chemistry, and depends on the change in a function proceeding at a rate proportional to its own magnitude.

VI.

Before concluding this paper it will be of interest to synthesize the knowledge that we have so far gained of the energy relations in photoreception. The two components of light are its intensity and the time of its action. The photochemical effect of each of these components has now been investigated, and quantitative expressions have been deduced for them. In addition we have studied the relation of these two variables to each other. Are the various findings consistent with one another, and can any additional information be gained by their combination?

We learned that for the minimum energy necessary to elicit a response the time and the intensity follow the Reciprocity Law of Bun-

sen and Roscoe (Hecht, 1919-20, *c*). This minimum photochemical effect results from the application of 5.62 meter candle seconds of energy, the intensity being inversely proportional to the time. Now that we know the individual photochemical effect of these two variables, we may determine whether their reciprocal relation obtains in the application of energy quantities greater than the minimum of 5.62 units. If the intensity is maintained constant and the time varied, the photolytic effect is directly proportional to the time (Hecht, 1918-19, *a*). This applies within an average range of approximately 50 meter candle seconds. If the Reciprocity Law holds for this range of energy values as well as for the minimum, the relation between the intensity factor and its photochemical effect should be the same, within the experimental error, as that found for the time factor. The photolytic effect of the light should thus be a linear function of its intensity factor within a range of 50 units of energy.

The broken line in Fig. 2 shows that this is true. The first three points in Fig. 2 cover a range of 408 meter candles. This gradation of intensity at an exposure of 0.133 second gives a variation of 54 units, similar to the range covered by the time factor. Within these 54 units the photochemical effect of the light is very obviously a linear function of the intensity, as the straight line in Fig. 2 shows. We may then conclude that the Reciprocity Law applies to the photoreception of *Mya* not only for the minimum energy requirement of 5.62 meter candle seconds, but for a range of 50 meter candle seconds as well.

The energy relations of the photoreceptor process therefore form a consistent scheme of things. This brings increased confidence in the results themselves as well as in their interpretation.

VII.

The investigation of the effect of intensity on the mechanism of photic sensitivity, with which this paper has been concerned, is of more than immediate interest. Most of the data on the photoreception of animals, particularly of higher vertebrates, are given in terms of intensity. Such data have heretofore been refractory to anything but a superficial treatment.

Among sensory processes in animals, the photic sensitivity of *Mya* is the only one that has so far yielded to physicochemical analysis. A broader application of the findings with *Mya* hinges to a large extent on the knowledge gained in the present investigation.

A concrete physicochemical mechanism has been proposed to account for photoreception in *Mya*. Fortunately the effect of the intensity on this mechanism has turned out to be a simple application of a general principle of physics and chemistry. We are therefore in a position to attack similar problems of perhaps wider interest to general physiology.

SUMMARY.

1. In the photosensory process of *Mya* the latent period varies inversely as the intensity of the stimulating light.

2. Quantitative analysis of the data shows that the photochemical effect of the light is a logarithmic function of its intensity, the two variables being related to each other according to the well known "compound interest" law.

3. Comparison with previous experiments demonstrates that the Reciprocity Law of Bunsen and Roscoe applies to the photosensory process not only for the minimum energy required for a response, but for a much greater range of energy application as well.

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LABYRINTH AND EQUILIBRIUM.

II. THE MECHANISM OF THE DYNAMIC FUNCTIONS OF THE LABYRINTH.

By S. S. MAXWELL.

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(Received for publication, January 23, 1920.)

In a previous paper,¹ I have shown that the ampullæ alone, without the otoliths, suffice for all the dynamic functions of equilibrium of the ear, and that the otoliths alone, without the ampullæ, also suffice for all the dynamic functions except that of response to rotation in a horizontal plane. I wish now to point out what I believe to be the mechanisms by which these reactions are brought about.

The Dynamic Functions of the Ampullæ.

Mach, Brown, and Breuer at first attached paramount importance to the space relations of the semicircular canals. It was assumed that rotation of the head in the plane of a canal caused, by the inertia of the endolymph, a current within the canal contrary to the direction of rotation. It was supposed that the hair cells of the crista were deflected by the current and stimulation of the nerve endings resulted. Mach,² however, very soon saw that under the conditions existing in the labyrinth such a current could not be produced, and Breuer later admitted that the hair cells do not project into the endolymph but are covered by a gelatinous mass.

On the experimental side, Loeb³ found that in the dogfish the canals could be cut through and even large portions could be excised without

¹ Maxwell, S. S., Labyrinth and equilibrium. I. A comparison of the effect of removal of the otolith organs and of the semicircular canals, *J. Gen. Physiol.*, 1919-20, ii, 123.

² Mach, E., Grundlinien der Lehre von den Bewegungsempfindungen, Leipsic, 1875.

³ Loeb, J., Ueber Geotropismus bei Thieren, *Arch. ges. Physiol.*, 1891, xlix, 175.

affecting the compensatory movements or the functions of equilibration, and Ewald⁴ stated that in the pigeon, after the canals had been ligatured, plugged, and cut, compensatory movements of the eyeballs and eye nystagmus were produced by rotation.

These experiments show that the canals are not necessary to the dynamic functions. Certain objections, however, might be raised. Loeb does not state specifically that all the canals were cut. Since I have shown that all the dynamic functions except that of response to rotation in a horizontal plane may be performed by an ear from which all the ampullæ have been removed, it would be necessary to know that the horizontal canals had been cut before the proof could be considered complete. Furthermore, in the dogfish each horizontal ampulla reacts to rotations in one direction only; this according to Ewald is not the case in the pigeon but his proof also is incomplete.

Since in the dogfish the response to horizontal rotation is brought about by the horizontal ampulla only, it would be a crucial experiment artificially to change the plane of this canal with reference to the skull of the animal and see whether this change does or does not alter the response to rotation. I have succeeded in doing this by the following method.

The right horizontal canal was laid bare for nearly the whole distance from its ampulla to the point where its posterior end reenters the vestibule. It was then ligatured and cut as far posterior as possible and the cut end was gently lifted into a vertical position, laid over against the skull, and supported there by a pledget of cotton. Its new plane was at right angles to its original plane and also at right angles to the long axis of the body. It is needless to say that in this operation extreme care must be taken not to exert the least traction on the ampulla. It is clear that with the canal in the new position rotation of the animal in a horizontal plane, that is around a dorsoventral axis, could not even theoretically give rise to a current in the canal. On rotation to the right, however, the eyes turn to the left and on rotation to the left the eyes turn to the right; that is, the ampulla whose canal is now at right angles to its normal position acts just like the

⁴ Ewald, J. R., *Physiologische Untersuchungen über das Endorgan des Nervus octavus*, Wiesbaden, 1892.

other ampulla whose canal is still horizontal. On the other hand, rotation of the animal around its longitudinal axis (in the new plane of the canal) never produces a deviation of the eyes to the left as it might be supposed to do if the rotation causes a current in the canal and the current excites the ampulla. This experiment, then, shows conclusively that the excitation of the sensory structures in the ampulla is due to some other cause than the production of a current in the canal.

Since no further consideration need be given to the possibility of currents in the semicircular canals as the cause of the excitation which on rotation gives rise to the reflex compensatory movements we may consider other possible causes. These might be (1) effects dependent on the inertia of the mass of liquid or other material in the vestibule, or (2) due to the inertia of the contents of the individual ampullæ, or (3) to inertia effects within the sensory cells themselves. It would be impossible to decide between these *a priori*.

In my earlier experiments I found that after destruction of the structures in the vestibule I could never obtain compensatory movements on rotating the dogfish around its dorsoventral axis. For a long time I was inclined to think that the absence of the reflex was due to some sort of injury to the ampullæ, although these appeared to be as sensitive as before to direct mechanical stimulation; the slightest pressure caused decided eye movements. When, however, I was finally able to remove the otolith from the recessus utriculi by slitting open the utriculus lengthwise without tearing it across I found that the compensatory movements to rotation in the horizontal plane were not abolished.¹ Since the destruction or the transection of the utriculus abolished the reflex with no apparent reduction in the direct sensitivity of the ampulla it became clear that the utricular (and possibly the saccular) structures are essential parts of the mechanism.

In attempting to analyze more closely the arrangements of the parts concerned it is to be noticed that the movement of rotation which acts as a stimulus to any given ampulla carries foremost the side of the ampulla which bears the crista. Thus the cristæ of the anterior canals are on the lower side of their ampullæ and a rotation of the head downwards excites them; the cristæ of the posterior canals are also on their lower sides and a rotation of the head upward (back part

of the head downward) excites them. So also the crista of the right horizontal canal is on its right or outer side and the stimulus for it is rotation to the right. Of course a similar relation exists for the left ampulla. Examination of the extensive series of drawings by Retzius⁵ shows that the dogfish is not a special case but that the arrangement is general.

A second fact which is significant is that the mouths of the ampullæ are continuous with the utriculus, an elongated, thin walled sac, stretched across the cavity of the vestibule and occupying only a portion, in the dogfish a not relatively large portion, of the vestibular space. Furthermore the utriculus is so attached by means of the sinus superior and other structures that a movement of the liquid contents of the vestibule might readily press it upwards towards the dorsal side of the cavity, but could have little effect to move it downwards. The relations as far as the ampulla of the anterior vertical canal is concerned are shown diagrammatically in Fig. 1. Rotation of the head downwards, that is, in the direction of the outer arrow, would tend by inertia to produce the same effect as if, with the head stationary, the perilymph was rotated in the opposite direction, as indicated by the small arrow within. This would put pressure and tension on the under side of the anterior end of the utriculus; this tension would be communicated to the ampulla and especially to its lower side which bears the crista.

In order to convince myself of the correctness or incorrectness of the above reasoning, I constructed a model by carving cavities and channels corresponding to the relations shown in Fig. 1. In these I placed a thin rubber model of the two canals shown in the figure. The canals and utriculus as well as the perilymphatic space were filled with mercury. On rotating the apparatus it could be seen that movement in one direction gave a very perceptible pull on the ampulla; movement in the opposite direction was almost without effect. It is possible that the rotation which puts the ampulla under mechanical strain would also tend to produce an increased liquid pressure within it, but this I could not determine in my present model.

⁵ Retzius, G., *Das Gehörorgan der Wirbelthiere. I. Das Gehörorgan der Fische und Amphibien*, Stockholm, 1882.

Careful dissection shows that mechanical relations analogous to those just described hold also for the posterior ampulla and the horizontal ampulla.

I wish to point out the advantage which the vestibular mechanism possesses on account of the mass of liquid. A relatively large free mass of liquid with a relatively small surface would show more inertia effect than a small mass with a relatively large surface area.

This principle was shown in a model made by Mr. W. F. Hoyt. Rossi⁶ had constructed a model of the size of a human semicircular

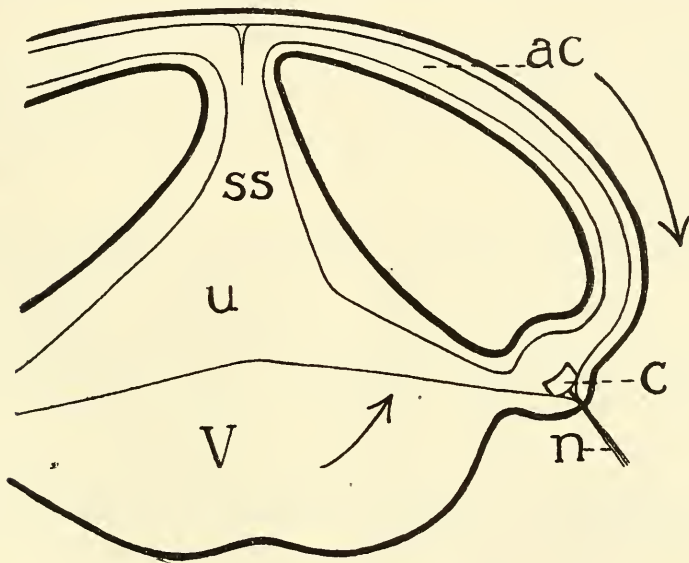


FIG. 1. Diagram to show relation of vestibular structures to ampulla; *v*, vestibule; *u*, utricle; *ss*, sinus superior; *ac*, anterior vertical canal; *c*, crista; *n*, nerve.

canal and ampulla and reported that movement of the liquid in the canal could be seen when the model was rotated. Hoyt made his model of glass for the greater transparency, and filled it with a liquid containing flakes of aluminium powder in suspension. When the model was rotated very rapidly and then suddenly stopped he could indeed see some movement in the canal, but the striking fact was that a marked movement of rotation took place in the ampulla.

⁶ Rossi, G., Di un modello per studiare gli spostamenti della endolinfa nei canali semicirculari, *Arch. Fisiol.*, 1914, xii, 349.

This movement was very much more readily produced and lasted much longer than that in the canal.

It has not been a part of the problem I have set myself to find explanations for the existence of structures in the labyrinth, but, on the contrary, to find out where and how definite functions are performed. Nevertheless nearly every person to whom I have communicated these results has asked, "What then are the functions of the semicircular canals?" It may be suggested that possibly a movement of liquid does take place through the canals, not as a stimulus to the nerve endings in the cristæ, but as a means of equalization of pressure. Indeed if the ampullæ were merely diverticula from the vestibule it is conceivable that pressure conditions could arise in them which might seriously affect their functioning. It is not unreasonable to suppose that the canals provide a means for the equalization of liquid pressure quite analogous to the use of the Eustachian tubes in equalizing air pressure.

The fact that in man and many mammals a nystagmus may be caused by irrigating the auditory canal with hot or cold water and that the character of this nystagmus differs for different positions of the head can be explained perfectly without assuming an unbelievable flow of liquid in the semicircular canals. If the temperature difference can cause convection currents in the inner ear at all under the conditions existing in such an experiment, it is certainly more reasonable to suppose that such movements of convection would arise in the mass of liquid contained in the vestibule than in the much smaller space of the canals where the friction would more readily overcome the tendency to movement. These considerations would not in the least invalidate the diagnostic use which Bárány has made of the phenomenon, but they do supply a rational explanation of its causation.

The Dynamic Functions of the Otolith Organ.

Breuer and others had suggested that the otoliths are concerned only with the static functions of the ear. Importance was attached to the space relations of the different otolith masses. But Parker⁷

⁷ Parker, G. H., Influence of the eyes, ears, and other allied sense organs on the movements of the dogfish, *Mustelis canis* (Mitchill), *Bull. Bureau of Fisheries*, 1909, xxix, 43.

and I each found that in the dogfish the large otolith of the sacculus has nothing to do with equilibrium; and I have shown that an ear from which the otolith of the sacculus as well as of all the ampullæ has been removed retains both static and dynamic functions. In this case the only part remaining which can mediate these functions is the small otolith organ in the recessus utriculi.

The otolith of the recessus utriculi is, in the dogfish, an oval or nearly circular mass, 3 or 4 mm. in diameter, shaped like a planoconvex lens. Its convex surface rests upon the corresponding concave surface of the macula in the bottom of the recessus. I have described it as resting on the macula, but the relation of its edges to the membranous walls suggests the idea that it is in reality partially suspended. To one who is actually performing these experiments it is a remarkably striking fact that all the functions performed by the ampullæ of the vertical canals can also be performed by this one organ. In the case of the ampullæ each one has a highly specialized function, responding to rotation in a single plane. The otolith organ on the contrary responds to rotations in all planes except the horizontal.

The approximation of the surfaces of the otolith and the macula, each almost perfectly spherical, suggests a mechanical arrangement by which any alteration of the position of the head would cause a corresponding change of pressure relations between the two structures. A pressure change of this sort could act as a stimulus to excite the compensatory movement appropriate to any particular rotation. Whether there is within the area of the macula a local differentiation of function comparable to that of the different ampullæ I have been unable to determine. The small size of the area to be explored and the difficulty of exact localization of an artificial stimulus have so far prevented an answer to this question.

STUDIES IN THE DYNAMICS OF HISTOGENESIS.

I. TENSION OF DIFFERENTIAL GROWTH AS A STIMULUS TO MYOGENESIS.

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(Received for publication, January 17, 1920.)

The prevalent opinion among embryologists in regard to the origin of muscular tissue is that of self-differentiation. This is due largely to the work of Wilson (1904) on *Dentalium*, and Conklin (1896-97, 1905) on *Cynthia* (*Styela*) *partita* and *Crepidula* in regard to the organ-forming elements of the cytoplasm, and to the experimental work of Harrison and Lewis (1904, 1905). Harrison ablated the spinal cord of the tadpole prior to the growth of the peripheral nerves into the limb buds. This operation eliminated any peculiar formation stimulus emanating from the nervous system. Still the differentiation of the contractile substance took place in the normal manner, as did the grouping of fibers into the individual muscles. Lewis (1910) draws the following conclusion based on Harrison's experiments, in regard to the genesis of cross-striated muscle:

"Thus it is seen that all the constructive processes involved in the production of the specific structure and arrangement of the muscle-fibres take place independently of stimuli from the nervous system and of the functional activity of the muscles themselves. Cross-striated muscle tissue and the individual muscles are thus self-differentiating."

The fact that there is considerable muscular differentiation before nerves establish a connection with their corresponding muscles has been shown by Bardeen (1900, 1906-07), Harrison, and Carey (1918) in the pig embryo. There is also considerable smooth muscle differentiation in the descending colon of the pig before either the myenteric or Auerbach's plexus is detected.

Lewis (1910) endeavored to solve the problem at how early a period in the development of the ovum this power of self-differentiation of muscle tissue begins. He found by transplanting tissue from the lips of the blastopore in the early gastrula stage of the frog that this tissue later on showed muscular differentiation. The conclusion is drawn "that muscle tissue is already predetermined in the early gastrula."

The idea conveyed by the last statement is that muscular tissue is formed, *sui generis*, by some inherent predetermination and not by the agency of its surroundings nor due to its position in the whole. Lewis' view-point is in accord with Conklin's (1905) as seen in the following statement of the latter observer: "The potencies or prospective values of any blastomere are not primarily a function of its position, but rather of its material substances."

There are three theories regarding cellular differentiation; first, the "mosaic theory" of Roux (1881), later modified by Wilson (1904), Conklin (1905), Zeleny (1904), and Boveri; second, the "organization theory" of Whitman and more recently elaborated by Child (1915) in his studies on metabolic gradients and individuality; third, "the homogeneity theory" of Driesch (1894, 1899). Driesch considers the peculiar organizing quality of protoplasm as due to the expression of a mysterious force wholly different from any in the inorganic world.

His, Roux (1881, 1892, 1893), Wilson (1892, 1893, 1897, 1904), and Conklin (1905) lay emphasis upon the cell as the key to all ultimate biological problems. Whitman, on the other hand, points out the inadequacy of the cell theory to development. "That organization precedes cell formation and regulates it, rather than the reverse, is a conclusion that forces itself upon us from many sides," is a summary of his studies. Morgan (1895, 1898) had deduced the idea from his studies on regeneration that the multicellular individual is a whole in the same sense that the unicellular form is a whole. Child (1899, 1915) also lays emphasis on the fact that it is the "organism—the individual, which is the unit and not the cell." Differentiation of a single cell, consequently, according to Child and Whitman, is a function of its position in the whole. This view is also upheld by Driesch (1894). Wilson and Conklin, on the other hand, conclude that potencies are functions of the material substance of the cell.

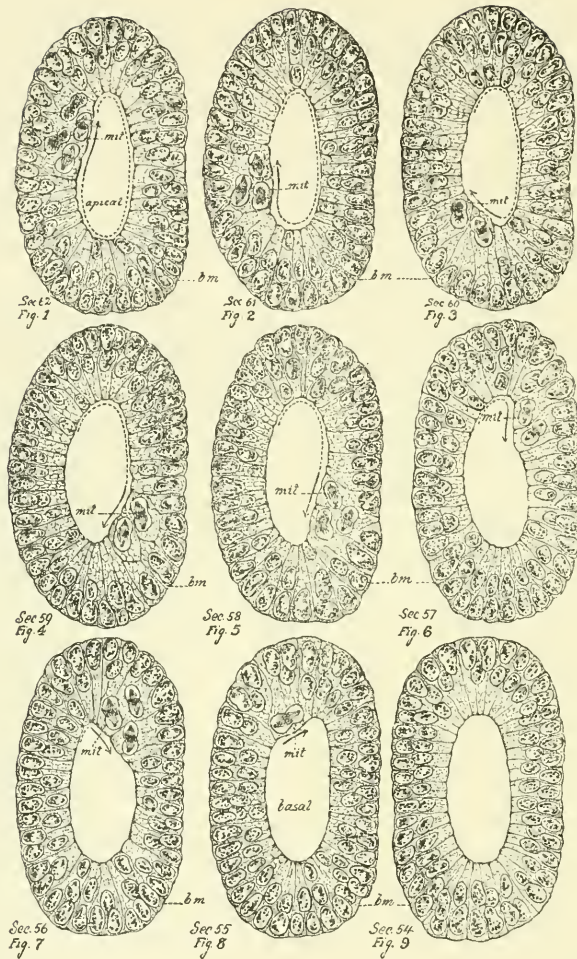
The influence of the organism as a whole in subjugating its dependent parts is convincingly shown by Loeb (1916) in his regeneration experiments on *Bryophyllum calycinum* and in his experiments on *Amblystoma* larva (1897). This influence is exerted through the blood stream by means of "hormones." The sound mechanistic attitude of Loeb toward development may be seen in the following statement: "As soon as we can show that a life phenomenon obeys a simple physical law there is no longer any need for assuming the action of non-physical agencies" (1916).

The object of this paper is not, however, to discuss historically the views of these various authors, but to emphasize certain facts in the development of muscle tissue hitherto overlooked. It is a well known fact that the embryo presents differential rates of growth. It is desired, therefore, to emphasize the fact that in embryological development there are zones of unequal or differential growth, and that the effects of these zones of growth are factors in histogenesis. The active and less active zones are defined with reference to the rate of cell division per mm. of cross-section. This principle was deduced from a series of studies on osteogenesis and myogenesis begun in 1914. Previous reports of a part of this work have been presented to the Association of American Anatomists (Carey, 1917, 1918, 1919).

It will be illuminating to search for the cellular forces outside of the immediate differentiating zone under observation. This search necessitates lower magnifications in order to enlarge our field of view. Heretofore, cytological differentiation has been studied *per se*, with magnifications of 1,000 to 2,000 diameters which considerably reduce our range of view. The higher magnifications are profitable in revealing cytological detail but the interpretation of the process is lost unless, in conjunction with the higher, intermediate magnifications are used. The employment of all magnifications of the microscope in connection with naked eye studies will reveal the interaction of related developing parts.

Early Development of the Descending Colon of the Pig.

The attention of the writer was directed to the fact, after plotting hundreds of intestinal epithelial mitotic figures, that these figures were usually confined to some definite region of the circumference of a single



FIGS. 1 to 9. Sections of the epithelial tube representing one complete turn in a dextrotropic rotation in a spiral manner of the mitotic division. The primary type is the left-handed helix. Spiral path is directed upward toward the ileocecal valve. Section 62 represents the head or apical end of the mitotic path. Sections 55 and 54 represent the tail or basal end of the mitotic spiral path. *mit*, mitosis; *bm*, basement membrane of the epithelial tube. Drawings are made with the aid of a Spencer Camera lucida. Sections 54 to 62 are from Pig Embryo 19, 24 mm. in length (Creighton Embryological Collection).

section (Figs. 1 to 9). This region was found to change at different levels of the serial sections. By graphic reconstruction this plot was found to form the path of a definite spiral. The predominant type was the left-handed helix. In one of the embryos of the twenty that were plotted this spiral was arranged as a right-handed helix (Figs. 1 to 9). The spiral itself presented a head or apical region in which mitotic figures were found to be numerous, and a tail or basal end in which there were fewer and fewer figures. The apical end of the spiral path is always directed towards the ileocecal valve and the basal end towards the rectum. Growth is, therefore, from below upwards in a spiral course. One spiral growth is quickly followed by a second which rifles a path slightly lateral to its predecessor. This in turn is followed by a third in a path still more lateral, and so on around the circumference. This intermittent rhythm of explosive spiral growth may be compared to that of the successive fire balls emitted by a roman candle in fireworks. The paths formed by this explosive spiral growth may be compared to those within the barrel of a Winchester rifle.

The most rapidly growing part of the intestine, therefore, is the epithelial tube. In embryos 10 to 25 mm. in length the descending colon grows relatively more rapidly in diameter than in length (Tables I and II). The increase in diameter is due primarily to the rapid growth of the entodermal epithelial tube and only partially to its surrounding mesenchymal cloak. The latter is relatively passive in growth with respect to the former (Fig. 10). It is during this early increase in diameter that the inner smooth muscle coat is in process of formation. The mesenchymal cells are drawn out gradually in a definite series of concentric rings. These rings appear not unlike those of the planet Saturn and the annular nebula in Lyra.

A definite centripetal force is active in the rapid spiral growth of the intestinal epithelial tube. The surrounding mesenchymal cells are thrown into an obvious series of concentric rings, according to their various densities. Those possessing the greatest density will join the outer ring in the tangential path of the force, whereas the inner ring will be composed of bodies forming a gradient of decreasing densities. The cells forming the outer ring will be most elongated.

TABLE I.

Measurements of Differential Growth of Descending Colon.

Length of embryo.	Thickness of mesenchymal wall.*	Diameter of epithelial tube.	Diameter of descending colon.	Epithelial tubular indices.†
<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>per cent</i>
10	0.085	0.048	0.218	6
12	0.099	0.069	0.267	8
13	0.115	0.075	0.305	8.5
14	0.128	0.081	0.337	10
16	0.126	0.089	0.341	14
19	0.124	0.095	0.343	18
20	0.123	0.099	0.345	20
22	0.122	0.119	0.363	25
23	0.121	0.138	0.383	30
24	0.120	0.152	0.392	38
25	0.115	0.164	0.394	40
27	0.109	0.188	0.406	44
30	0.104	0.208	0.416	46
32	0.099	0.220	0.418	47
35	0.098	0.246	0.442	48
37	0.096	0.260	0.452	50
39	0.093	0.279	0.465	52
40	0.092	0.289	0.473	54
42	0.090	0.312	0.482	57
45	0.083	0.321	0.486	61

* The mesenchymal wall begins to diminish in thickness after it reaches a width of 0.128 mm. in the 14 mm. stage of the pig embryo. This diminution is due to the tension caused by the more rapid epithelial tubular growth in diameter. Measurements made with B. and L. filar micrometer, calibrated.

† The ratio of the square of the mean diameter of the epithelial tube to that of the surrounding mesenchyme is referred to as the epithelial tubular index. It has been calculated from the following formula.

$$\left\{ \frac{\frac{(x+y)^2}{(2)} \times 100}{\frac{(X+Y)^2}{(2)} - \frac{(x+y)^2}{(2)}} \right\} = Z$$

x and y are the long and short diameters of the epithelial tube respectively. X and Y are the long and short diameters of the surrounding mesenchymal tube. Z is the ratio of the epithelial tube to the mesenchymal tube.

As this concentric initial smooth muscle layer becomes differentiated it tends to restrict the diametrical growth of the epithelial tube. The epithelial mitotic figures under this restriction shift their planes of division from a right angle to a parallel position with the smooth muscle cells. This shifting results in an elongation of the intestine.

TABLE II.

Ratio of Diameter to Length of Entire Colon.

Length of embryo.	Diameter of descending colon.	Length of entire colon.	Ratio of diameter to length of entire colon.
<i>mm.</i>	<i>mm.</i>	<i>mm.</i>	<i>mm.</i>
10	0.218	1.95	1: 9.9
12	0.267	2.00	1: 7.5
13	0.305	2.02	1: 6.6
14	0.337	2.05	1: 6.0
16	0.341	2.10	1: 6.1
19	0.343	2.20	1: 6.4
20	0.345	2.30	1: 6.7
22	0.363	2.75	1: 7.5
23	0.383	3.00	1: 7.8
24	0.392	3.50	1: 8.9
25	0.394	3.95	1: 10.0
27	0.406	6.00	1: 14.0
30	0.416	8.00	1: 19.0
32	0.418	11.00	1: 26.0
35	0.442	16.95	1: 36.0
37	0.452	18.00	1: 39.0
39	0.465	21.00	1: 45.0
40	0.473	23.00	1: 48.0
42	0.482	27.00	1: 56.0
45	0.486	29.00	1: 59.0
Adult.	50.000	7,000.00	1: 140.0

In embryos 25 to 40 mm. (Tables I and II) in length, the elongation of the descending colon is more rapid in growth than that of the diameter. It is during this period that the outer longitudinal muscular coat is in the process of formation. The rapid growth of the epithelial tube in length tends to elongate the peripheral undifferentiated mesenchymal cells which were not directly involved in the formation of the inner smooth muscular coat (Fig. 11).

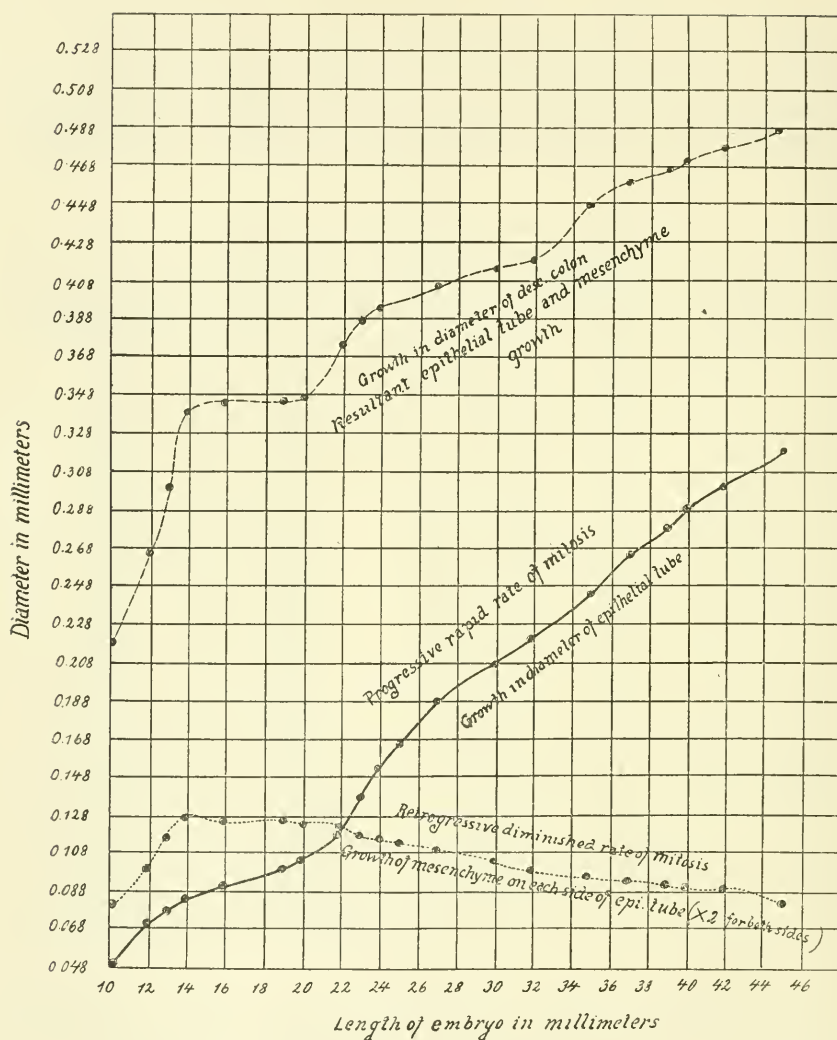


FIG. 10. Curves of differential growth of descending colon. The active growth of epithelial tube is contrasted with the passivity of the mesenchymal wall. An absolute decrease in thickness of the mesenchymal wall is seen after the stage of the 14 mm. pig embryo.

The differentiation of the outer longitudinal muscle coat therefore coincides, in time, with the rapid growth in length of the intestinal epithelium. The inner smooth muscle coat, on the other hand, is formed during the period of the rapid growth of the intestinal epithelial tube in diameter. Once the formation of the inner circular muscular rings is fairly established a resistance to growth in width is

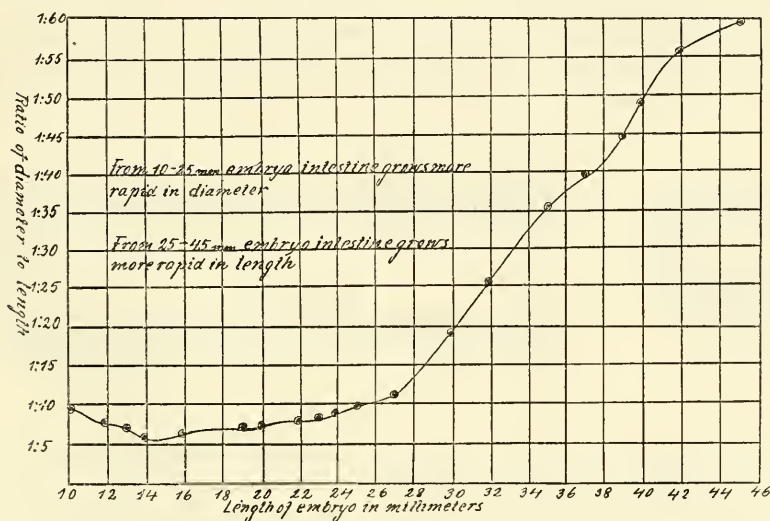


FIG. 11. Curve representing the ratio of the diameter to the length of the descending colon in embryos 10 to 45 mm. in length. Particular attention is directed to the fact that in embryos 10 to 25 mm. in length the intestine grows relatively more rapidly in diameter than in length. In embryos from 25 to 45 mm. in length the intestine grows more rapidly in length than in diameter. The inner circular smooth muscle is formed during the period of rapid growth of the intestine in diameter. During the period of the rapid growth of the intestine in length the histogenesis of the outer longitudinal smooth muscle coat is taking place.

encountered by the cells surrounding the rapidly dilating lumen. These cells then grow primarily along the path of least resistance in a longitudinal manner. At this stage the longitudinal muscle cell, spherical in shape (Fig. 12), is elongated to a spindle-shaped structure (Fig. 13).

An interesting correlation in the development of the esophagus in man may be cited. This correlation was detected in the work of

Jackson and in that of Keibel and Elze. The former investigator studied the developmental topography of the esophagus, the two latter the histogenesis of the esophagus. Jackson states that the descent of the stomach is accompanied by a great elongation of the esophagus. In a 9.4 mm. specimen, the esophagus measures 1.8 mm. At this proportion it should measure 4.3 mm. in a 22.8 mm. embryo but its actual length is found to be 8 mm. A year previous to this, Keibel and Elze reported that the esophagus in 12.5 mm. embryos shows a circular but no longitudinal muscle layer. In 17 mm. embryos, they find a circular layer with the longitudinal layer faintly

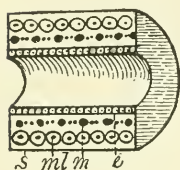


FIG. 12.

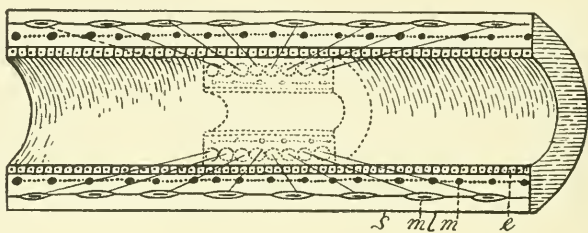


FIG. 13.

FIG. 12. Longitudinal section of intestine; *s*, peritoneal epithelium; *ml*, mesenchyme; *m*, circular muscle; *e*, epithelium.

FIG. 13. Longitudinal section of intestine schematizing the elongation of the intestine represented in Fig. 12. Due to the resistance of the inner smooth muscle layer *m*, the intestinal epithelium grows in the longitudinal path of least resistance. This results in the elongation of the outer mesenchymal cells *ml* (Fig. 12) into the elliptical or spindle cells *ml* (Fig. 13).

indicated. The histogenesis of the outer longitudinal layer of the esophagus as studied by Keibel and Elze coincides in time with the rapid elongation of the esophagus, due to the descent of the stomach, as recorded by Jackson.

Interpretation of Results.

The result of the action of a force on an elastic body is the production of a strain. If mechanical forces are at work on organic matter, they tend to produce similar results to these acting upon inert matter. Too frequently the term self-differentiation is applied to alteration of

form and internal structure of developing cells without searching the immediate environment of the specializing cells or syncytium to ascertain whether or not these changes are attributable to forces outside of the differentiating zone. This applies particularly to the differentiation of bone and muscle tissue. If a cell changes in form successively through the spherical, ellipsoid, and spindle stages it undergoes a strain. A strain is usually due to an external force which elicits internal reacting stresses in the body acted upon. Cytological differentiation is frequently a manifestation of these internal reacting stresses to forces extrinsic to the differentiating zone.

A strain is produced in certain regions of the embryo by the expansion of a rapidly dividing group of cells against a less active or relatively passive group of cells. After their differentiation the relatively passive group of cells in their turn react upon the former. This action and reaction are objectively evident by a retardation or alteration of the rate of growth, or by a change produced in the external form or internal structure of the cells involved.

In this study, the initial zone of rapid growth is found in the epithelial tube. The rapid spiral expansion of the entodermal epithelial tube reacts against the surrounding splanchnic mesenchyme with the result that the less actively growing cells of the peripheral region of the intestinal wall are elongated. Later the elongated, differentiated mesenchymal cells cause a retardation of the growth in diameter of the epithelium. Immediately following this retardation of diametrical growth the period of rapid growth in length of the intestine takes place. In this development, therefore, the influence of unequal growth zones is definitely shown as furnishing a tensional stimulus for the differentiation of muscle.

This action is diagrammatically illustrated in Figs. 14 and 15. In Fig. 15 the growth in diameter of the intestine is schematized; the rapid increase in width is shown as due primarily to the increase in the lumen. This growth is due to rapid mitotic activity of the epithelium (*e*, Fig. 14, to *e'*, Fig. 15). In the lumen of Fig. 15, the former is represented in a spiral manner, *sg*. In this growth the strain upon the surrounding mesenchymal cells *m* is illustrated. These cells are strained by the external applied forces of the progressively diverging radii. The internal reacting stresses are manifested by the changes

in shape through spherical m , ellipsoidal m' , and spindle m'' cellular phases in Fig. 15. In addition to the homogeneous strain to which the cells m , m' , and m'' are subjected there is a definite pressure exerted by the epithelial cells lining the expanding lumen.

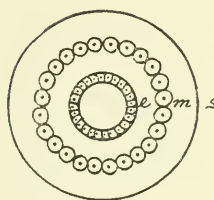


FIG. 14.

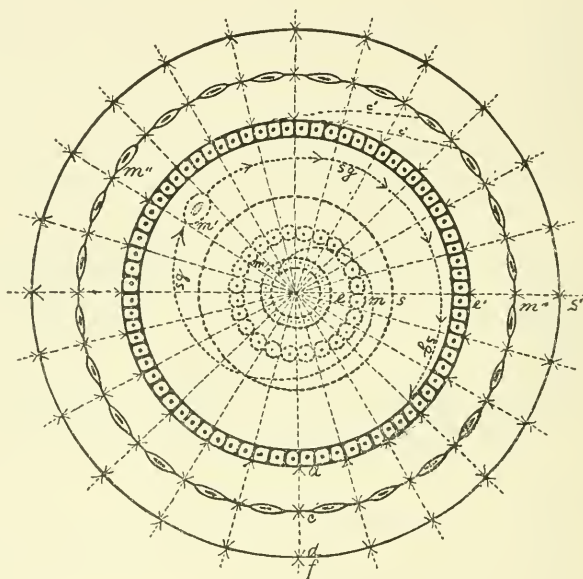


FIG. 15.

FIG. 14. Transverse section of intestine; e , epithelium; m , mesenchyme (spherical nucleus); s , peritoneal epithelium.

FIG. 15. Transverse section of intestine grown to three times the width represented in Fig. 14. Fig. 15 is represented in broken lines within the lumen. The spiral growth of the epithelium is represented by the broken lines sg . The tension, upon the mesenchyme, by the most rapidly growing epithelium, is shown in the elongated muscle cells m'' . These cells are homogeneously strained in the centrifugal path c' due to the progressively diverging radii. Cells marked m , m' , and m'' represent the progressive steps in the strain ellipsoid in the differentiation of a muscle from a mesenchyme cell. The expansile force of the epithelium is shown by the double arrow $a-d$; the reacting resistance of the serous membrane by the line $d-a$. Equilibrium is established in the middle of the mesenchyme and is graphically represented by the double arrows $a-b$ and $d-c$. This is another factor in the tensional elongation of the middle cells. The smooth muscle ring exerts a centrifugal reaction to the applied centripetal force of the dextrotropic spiral rotation of the epithelial tube. The mitotic figures of the descending colon primarily follow the path of a left-handed helix.

This force of expansion is represented by the arrow $a-d$. A resistance f , due to the peritoneal epithelium, is met. This causes a reaction $d-a$. With progressively increasing growth a zone of equilibrium of expansile and reacting forces is established in the middle, represented by the double arrows $b-a$, and $d-c$. This action and reaction of forces is another factor tending to compress the cells in the middle of the mesenchymal wall of the intestine. The action of the centripetal component of the spiral growth of epithelium in forming the rings of dense spindle-shaped muscle cells m'' is represented by the broken curved arrows. The spiral growth of the epithelial tube in a dextrotropic rotation exerts a centripetal force upon the surrounding mesenchyme. The mesenchyme consequently exerts a simultaneous equal and opposite centrifugal force upon the epithelial tube. This growth is primarily in the form of a left-handed helix from the rectal to the ileocecal valvular regions of the large intestine. In Figs. 14 and 15 the right-handed helix is depicted.

Although by direct observation of serial sections no motion is seen, there is, however, objective evidence of homogeneous and ellipsoidal strains upon the surrounding mesenchyme. The mesenchyme is drawn out into concentric rings, the outermost of which are most viscid, by the spiral growth of the epithelial tube, roughly comparable to the increase of viscosity and concentric annular formation of egg albumin when subjected to an egg-beater.

CONCLUSION.

The genesis and maintenance of muscle tissue represents a resultant or equilibration of converging factors which are active and formative during development. *One of these factors is the tensional stresses to which the mesenchyme is subjected by a force extrinsic to the differentiating zone.* In subsequent involution or degeneration of muscular tissue during the prenatal or postnatal periods, this equilibrium is upset by altering or destroying the tensional reacting stress.

Tension is developed when a muscle contracts. Contractility is a fundamental property of protoplasm and, when manifested, tension is developed. In both, the development and specific function of muscle tissue, tensional stresses are inseparably involved. The

Ameba possesses the property of contractility in all possible directions. The function of contraction in one definite direction characterizes muscle tissue from that of undifferentiated protoplasm. What initiates the progressive series of physicochemical changes in the mesenchyme resulting in an alteration of its attribute from non-specificity to its specificity of direction of contractility? This question is answered as follows.

The mesenchyme before differentiating into muscle tissue must be subjected to a certain minimal homogeneous and ellipsoidal strain. This strain is objectively evident by an alteration of the form of the spherical nuclei, into the ellipsoidal and spindle conditions and by an elongation of the granular cytoplasm into parallel granular and continuous fibrillæ. The fibrillæ are arranged along lines of internal and reacting tensional stresses. The ends of the mesenchymal cells, in tension, must be attached to supports of which one, at least, is mobile. The tensional stresses are reactions to simultaneous forces extrinsic to the zone of myogenesis. The external forces are produced by a progressive divergence or separation of the mobile supports to which the mesenchymal cells are attached. Therefore, muscle tissue is not self-differentiating but is dependent upon an external dynamic stimulus. As regards smooth muscle this stimulus is the *tension of differential growth*.

SUMMARY.

1. The region of most active mitosis per mm. of cross-section in the intestine is the entodermal epithelial tube. The mitotic figures primarily follow the path of a right-handed helix. In one of the twenty embryos the mitotic figures describe the path of a right-handed helix.
2. The region of least active or relatively passive growth per mm. of cross-section is the mesenchyme, derived from the splanchnic mesoderm surrounding the epithelial tube.
3. The rapid expansion, due to epithelial growth in a rotating spiral manner, of the intestinal lumen is greater than the activity manifest in the surrounding mesenchyme. This causes a pressure in the latter resulting in a flattening and elongation of the mesenchymal cells. The successive changes in shape of these cells through the spherical, ellipsoidal, and spindle cellular phases are seen. The

mesenchymal wall decreases in thickness, due to tension caused by epithelial tubular dilation.

4. The rotating spiral growth of the epithelial cells causes the formation of a series of mesenchymal cellular and fibrillar concentric rings due to the centripetal force of the former.

5. The circular, smooth muscle cells are differentiated in the outer, more condensed margins of the ring. At these points the developing tensional stresses are greater than within the ring.

6. The inner circular smooth muscle coat is the first one differentiated and is incident to the rapid growth of the epithelial tube in diameter. The former soon tends to restrict the growth of the epithelial tube in diameter. The tube, pursuing the lines of least resistance, grows in length. During the period of rapid growth in length the outer longitudinal muscle coat is in the process of formation.

7. The tensional stresses to which the elongated strained mesenchymal cells are subjected appear to be a dynamic stimulus to smooth muscle differentiation.

8. From this study of a closely graded and progressive series of sections of intestinal development, the conclusion is drawn that muscle tissue is not self-differentiating, in the strict sense of the term, but that the *tension of differential growth* acts as the stimulus to smooth muscle differentiation.

The writer wishes to express his indebtedness to Professor H. von W. Schulte for his interest and his valuable suggestions; to Madame Helen Ziska for the illustrations; and to his wife for her help in reading the proof.

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THE NATURE OF THE DIRECTIVE INFLUENCE OF GRAVITY ON THE ARRANGEMENT OF ORGANS IN REGENERATION.

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(Received for publication, January 16, 1920.)

I.

In preceding papers it has been shown that gravity has a directive influence on the arrangement of certain organs in the regeneration of *Bryophyllum calycinum*. Thus, when an excised piece of stem of *Bryophyllum* is suspended horizontally in moist air, roots will grow abundantly on the lower side of the stem but not on the upper side, with the exception of the basal end where the roots will grow on both sides. In order to get abundant root formation it is necessary to preserve one of the apical leaves on such a stem since the leaf furnishes most of the material for the growth of the roots.¹

A second case in which this directive influence shows itself is the leaf of the same plant. When we suspend an isolated leaf of *Bryophyllum* (in moist air) sidewise, in a vertical plane, roots and shoots will grow abundantly in the notches of the lower side, but less abundantly and frequently not at all in the notches of the upper side of the leaf (Fig. 1, upper row). This phenomenon was explained by the writer in the following way. Gravity causes a slightly greater collection of sap on the lower side of the organs mentioned and this causes the dormant buds for root formation to grow out a little earlier or more quickly on the lower than on the upper side of the stem or the leaf. It is a general rule in the phenomena of regeneration in *Bryophyllum* that organs which grow out first or more quickly attract for some reason the flow of sap (possibly by modifying the direction of the sap flow) and thereby inhibit or retard the growth of similar organs in other places, and this inhibitory factor is added to the influence of

¹ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 687.

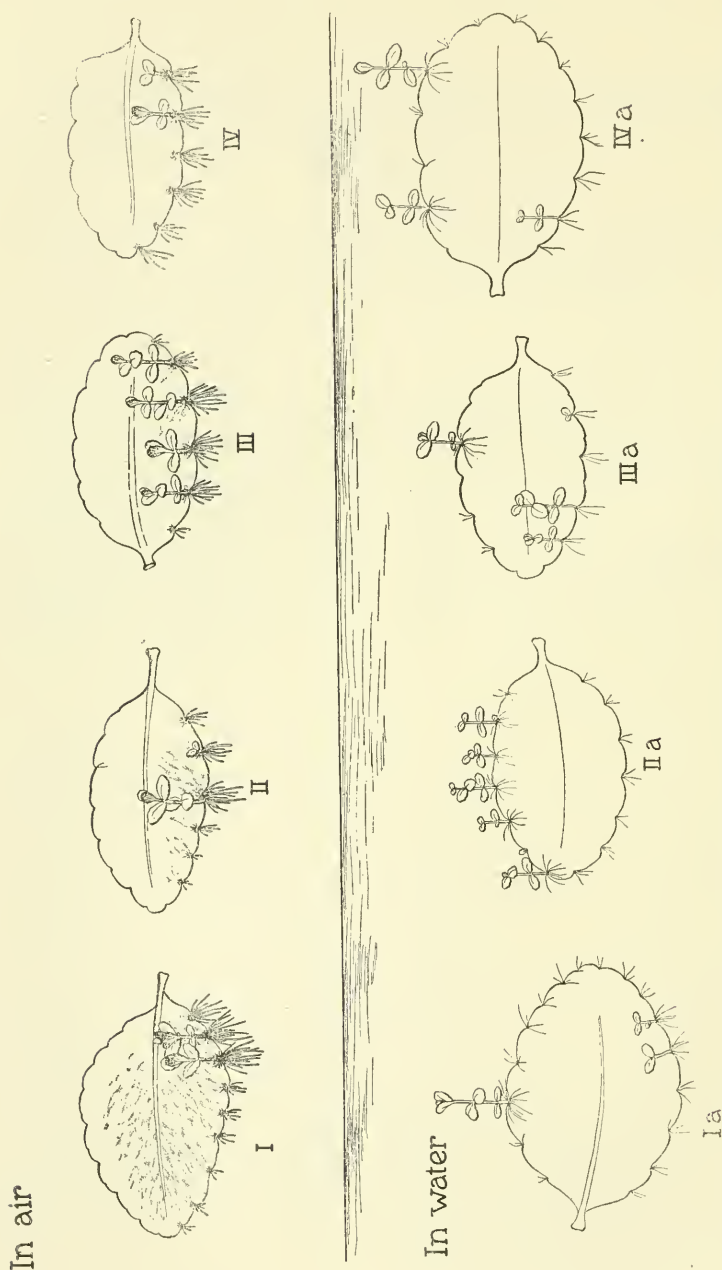


FIG. 1. Upper row, leaves suspended in air, lower row, leaves suspended in water. In moist air (upper row) roots and shoots are found only in the notches of the lower side of the leaves, while in water this directive influence of gravity does not exist, inasmuch as roots and shoots are formed on both the upper and the lower sides of each leaf. Only the leaves in the upper row show the red pigment indicated by stippling. Duration of experiment, Oct. 26 to Dec. 15, 1919.

gravity upon the distribution of sap. It is, therefore, not gravity alone which determines the directive effect in these cases but gravity in combination with the modification of the sap flow towards a growing organ.¹

This reasoning is supported by the following facts. When we prevent the growth of roots and shoots on the lower side of the leaf by cutting off the dormant buds contained in the notches of the lower side of a leaf of *Bryophyllum* suspended vertically and sidewise, roots and shoots will now develop as abundantly on the upper side as they otherwise would have developed on the lower side; the only difference being that the roots begin to appear on the upper side slightly later than they would have appeared on the lower side.¹

The same proof can be furnished for the formation of roots on the lower side of a horizontally placed stem. When we cut off the lower half of such a stem the roots will now form abundantly on the upper side of the stem.¹

The fact that some sap collects on the lower side of a piece of stem suspended horizontally or on the lower side of an isolated leaf suspended sidewise in a vertical plane may be compared to the behavior of edematous liquid in animals which also follows gravity. It is possible that only that part of the sap of an isolated organ is thus affected by gravity which is not in active circulation in the vessels. The circulation in the vessels will cause an abundant collection of material at the ends of a piece of stem around the whole circumference of the piece and this may account for the fact that at the basal end of an excised piece of stem suspended horizontally in air roots develop on both the lower and upper side in such abundance that the slight quantity of sap collected through the influence of gravity on the lower side of the base becomes a negligible factor.

The real share of gravity in the directive influence on root and shoot formation is, therefore, a modest one since it needs consist only in a slightly greater collection of sap on the lower side of an organ, just sufficient to accelerate the growth of roots on the lower side.

It occurred to the writer that this conception of the directive influence of gravity on root formation might be tested in still another way; namely, by suspending leaves or pieces of stem under water. In this case the influence of gravity on root formation should dis-

appear or be greatly diminished, since the influence of the water furnished from the outside might tend to make the influence of the slight quantity of sap collected on the lower side through the influence of gravity a negligible factor.

When we suspend pieces of stem of *Bryophyllum* horizontally under water (instead of in air) roots no longer develop on the lower side only but on the upper side as well (Figs. 2 and 3). The sap will in this case collect also in somewhat greater abundance on the lower side of the stem, but this will cause no retardation of growth on the

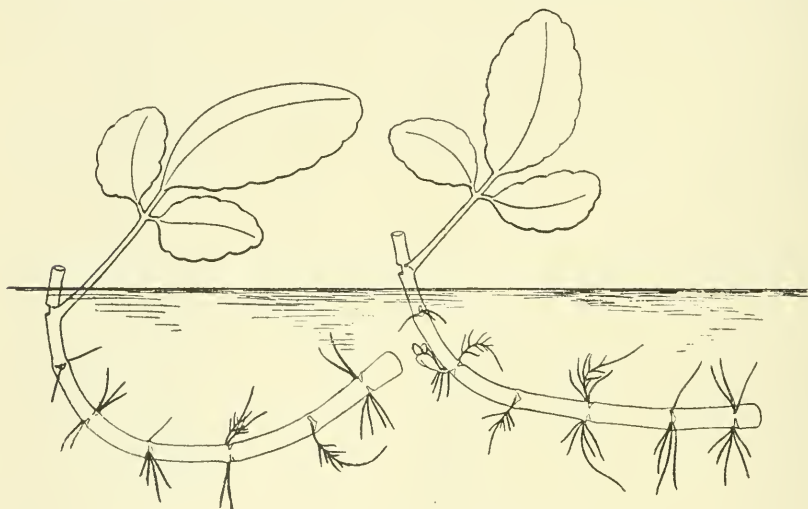


FIG. 2. Stems suspended horizontally under water. Roots form no longer exclusively on the lower side of the stems as they would have done had the stems been suspended in air, but on both the upper and lower side of the stem. The stems were originally straight and the curvature is due to geotropism; *i.e.*, greater growth on the lower side.

upper side on account of the abundance of water on the upper side of the stem. This is also true for leaves. When we suspend leaves vertically and sidewise under water the roots and shoots will develop equally fast on the upper and lower sides of the leaf and the influence of gravity will disappear (Fig. 1, lower row). In both cases it is necessary to suspend the organs near the surface of the water so that their oxygen supply will not suffer too much.

We can finally submit our idea concerning the nature of the directive effect of gravity upon the arrangement of organs to a quantitative test. When an organ (*e.g.* a leaf suspended vertically and sidewise



FIG. 3. Same as Fig. 2. Duration of experiment, Oct. 13 to Nov. 1, 1919.

in moist air) forms roots or shoots exclusively on the lower side, the material for these new organs must be partly withdrawn from the upper side of the organ and we must be able to prove that the dry

weight of the lower half of such an organ is always greater than the dry weight of the upper half; while when an organ forms shoots and roots on both the upper and lower side (e.g. a leaf suspended vertically and sidewise in water) no such difference in the dry weight of the upper and lower half should be expected. Determinations made by the writer confirm this expectation.

These experiments were carried on in the following way. A number of leaves were suspended vertically and sidewise in an aquarium

TABLE I.

Dry Weight of Upper and Lower Halves of Leaves Suspended Vertically and Sidewise in Air. Roots and Shoots on Lower Side Only.

Duration of experiment.		Dry weight.	Ratio dry weight upper halves dry weight lower halves
<i>days</i>		<i>gm.</i>	
A. 22	Upper halves of six leaves.	0.620	0.815
	Lower " " " "	0.751	
B. 32	Upper " " " "	0.810	0.800
	Lower " " " "	1.011	
C. 36	Upper " " " "	0.570	0.888
	Lower " " " "	0.642	
D. 35	Upper " " eight "	0.582	0.855
	Lower " " " "	0.681	
E. 31	Upper " " five "	0.499	0.872
	Lower " " " "	0.572	

filled with moist air. The majority of these leaves formed roots in abundance on the lower side but not on the upper side. For this experiment leaves with perfect symmetry were selected. After about 4 or 5 weeks a number of such leaves were cut as accurately as possible along the middle rib and the fresh and dry weights of the upper and lower halves were determined. The dry weight of roots and tiny shoots formed on the lower side was included in the dry weight of the lower halves of the leaves. The lower halves of the leaves had, without exception, a higher dry weight than the upper halves (Table I).

As a control the same experiments were made with leaves suspended under water which had formed roots and shoots on both their upper and their under sides though there were fewer on the upper side. In this case there was no constant difference between

TABLE II.

Controls, Dry Weight of Upper and Lower Halves of Leaves Suspended Vertically and Sidewise in Water. Roots and Shoots on Upper and Lower Sides.

Duration of experiment.		Dry weight.	Ratio dry weight upper halves, dry weight lower halves
<i>days</i>		<i>gm.</i>	
A. 29	Upper halves of six leaves with ten shoots. Lower " " " " " eleven "	0.561 0.538	1.04
B. 32	Upper " " five " " four " Lower " " " " " two "	0.672 0.606	1.11

TABLE III.

Dry Weight of Upper and Lower Halves of Leaves Suspended Vertically and Sidewise in Moist Air, Which Formed Some Shoots on Both Upper and Lower Sides.

Duration of experiment.		Dry weight.	Ratio dry weight upper halves, dry weight lower halves
<i>days</i>		<i>gm.</i>	
35	Upper halves of four leaves. Lower " " " "	0.365 0.369	0.990
31	Upper " " seven " Lower " " " "	0.735 0.783	0.940

the dry weights of the upper and lower halves. The differences found were very slight and occurred in both directions (Table II).

The same was true for leaves raised in moist air, which formed some shoots on the upper side. The dry weights of the mass of the upper and lower halves did not differ (Table III).

II.

The idea that a collection of sap occurs in the lower parts of a leaf suspended in a vertical plane can be demonstrated through the fact that leaves thus suspended in moist air form a reddish or purple pigment which has a tendency to collect in the lower parts of such a leaf. Fig. 4 shows two leaves with this pigment. The leaves had been suspended in moist air from April 17 to May 14, 1919. The

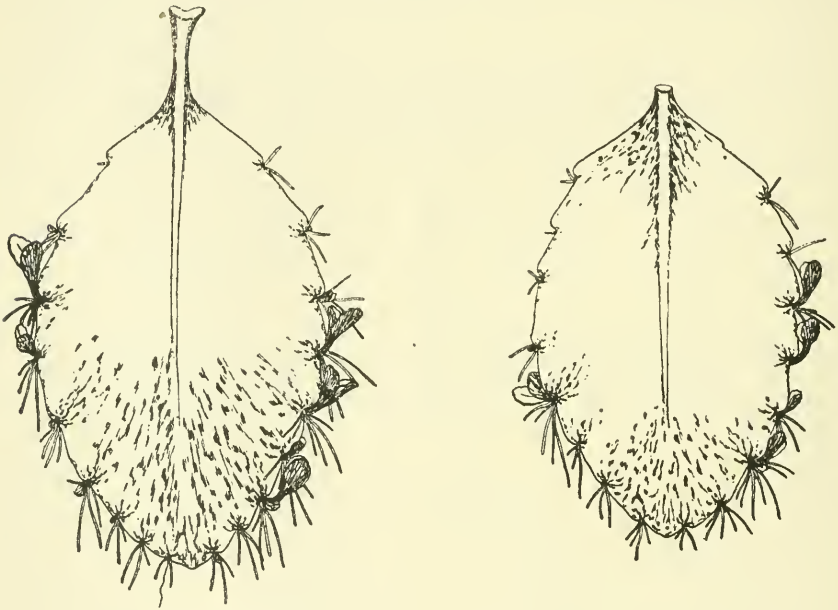


FIG. 4. Leaves suspended in moist air. Collection of reddish or purplish pigment in lower halves of the leaves, following the vessels. It is also obvious that the pigment collects more densely near each notch where a shoot or roots grow out. The leaves of the new shoots formed are full of pigment while the roots are free from it. Duration of experiment, Apr. 17 to May 14, 1919.

reddish or purple pigment is indicated in the drawing by black stippling. It is obvious that the pigment collects in the lowest parts of the leaf, that it follows the vessels, and that it also flows into the young leaves, thus supporting the view that a deflection of the sap flow towards the new shoots occurs in such a leaf. It does not,

however, collect in the new roots. A closer inspection of the leaves shows that the red pigment collects not only in the lower half of the leaves but also in those notches of the upper part of the leaf where roots or shoots are growing vigorously, thus supporting the view that the flow of sap is directed to rapidly growing organs. While a small amount of red pigment may possibly be visible in a leaf under normal conditions (especially along the edge of a young leaf), a noticeable amount is formed when the amount of water in the leaf is diminished.

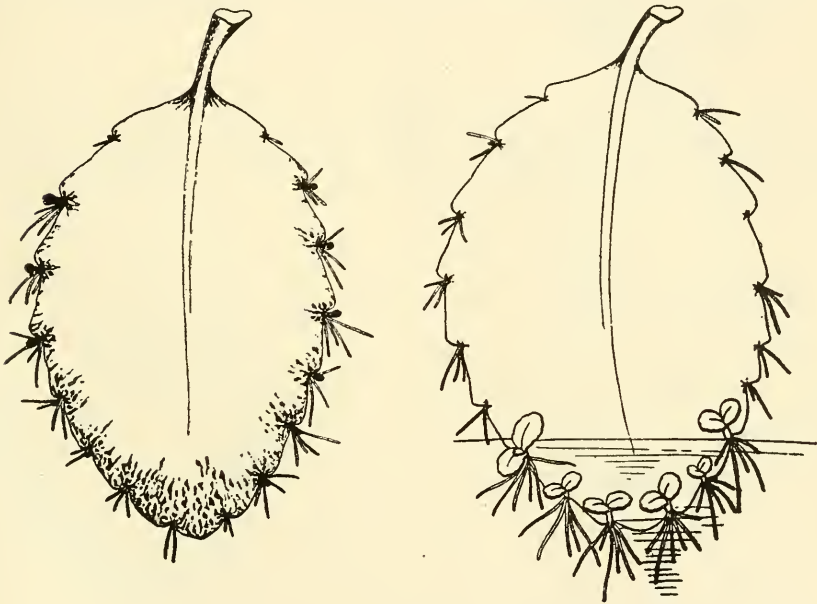


FIG. 5. Two sister leaves, one suspended in moist air, the other dipping with its apex into water. Only the former shows the pigment formation while the latter does not. This result is general and proves that the formation of the reddish pigment is favored by a diminution in the amount of water in the leaf since in the leaves suspended in moist air the ratio of dry to fresh weight is greater than in leaves which dip into water. Duration of experiment, Apr. 2 to Apr. 17, 1919.

This is shown by a comparison of the two sister leaves in Fig. 5, one of which (the one on the right) dipped with its apex into water while the other (the one to the left) was suspended in moist air. The ratio of dry weight to fresh weight of such leaves is always greater in the

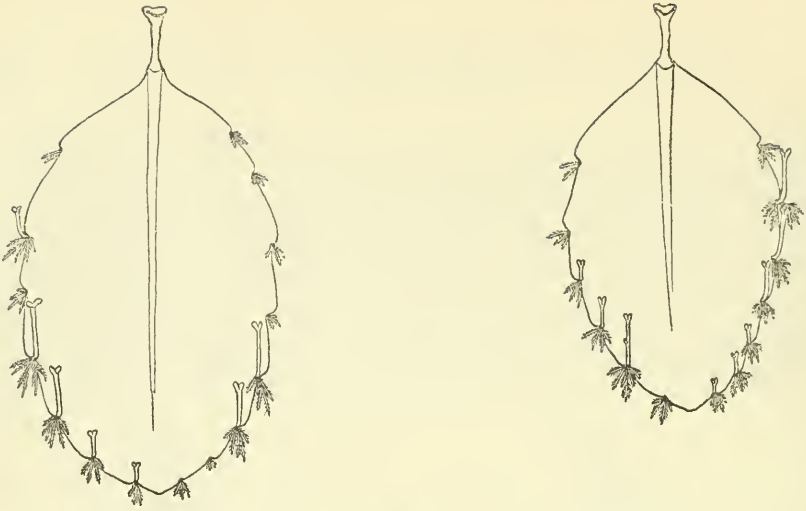


FIG. 6. Isolated leaves suspended in moist air but kept in dark. Although roots and shoots are formed no red pigment is noticeable. Duration of experiment, Apr. 20 to May 13, 1919.

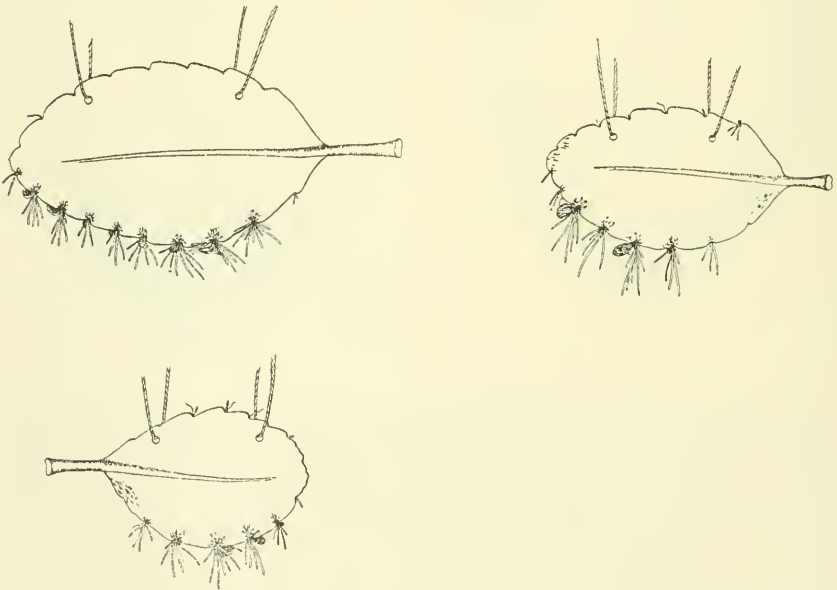


FIG. 7. Leaves suspended sidewise and in a vertical plane in moist air. The purple pigment collects near the notches on the lower side where roots and shoots develop. The young leaves are full of this purplish pigment. It collects also on the upper side of the middle rib while the lower side of the middle rib is free from pigment. In the petiole pigment exists on both the lower and upper side.

leaves suspended in air than in water. The leaf dipped into water (*i.e.* the leaf with a normal ratio of dry to fresh weight) formed no noticeable quantity of pigment while the other leaf with less water formed a considerable quantity. This difference is constant. A second factor necessary for the appearance of red pigment in the leaf is light. When leaves are suspended in moist air but kept in the dark they form no noticeable amount of red or purple pigment (Fig. 6) though they form roots and shoots.

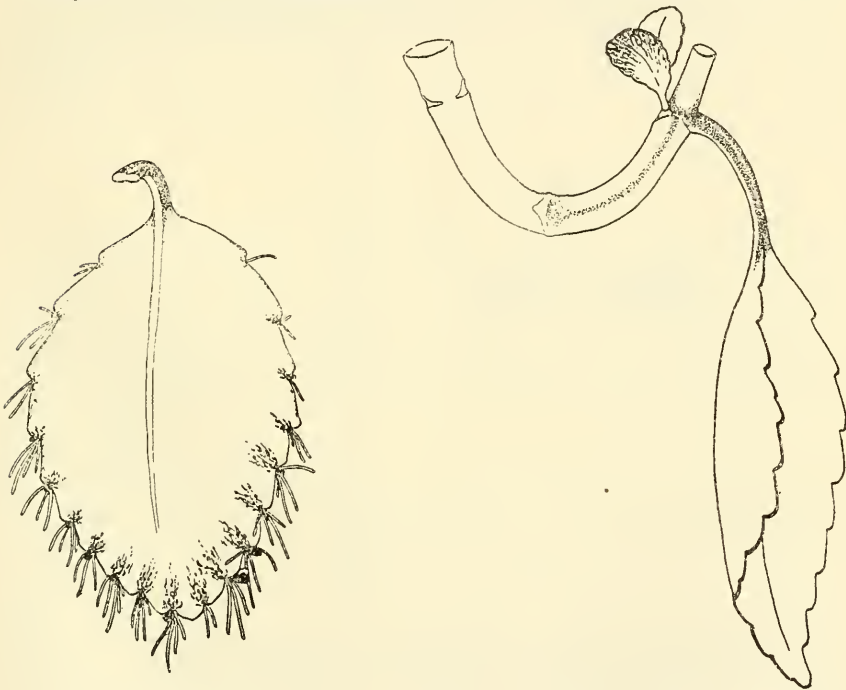


FIG. 8. Two sister leaves, one (to the right) connected with a piece of stem, the other (to the left) detached from the stem. Though both leaves are suspended in moist air only the detached (left) leaf is full of pigment, while the leaf in connection with stem is free from the purple pigment, which collects in the stem and in the leaves of the bud formed by the stem.

The most important fact for our problem is the distribution of sap in leaves suspended sidewise and in a vertical plane in moist air. In such leaves a collection of sap is found in the vessels close to the notches of the lower side of the leaves where the roots and shoots develop (Fig. 7).

We have stated in a preceding paper² that the sap from a leaf flows normally into the stem and that this is the reason of the inhibitory or retarding influence of the stem upon root and shoot formation in a leaf of *Bryophyllum*. This idea is supported by the fact that when a piece of stem is left in connection with a leaf suspended in moist air no collection of red or purplish pigment occurs in the leaf but that

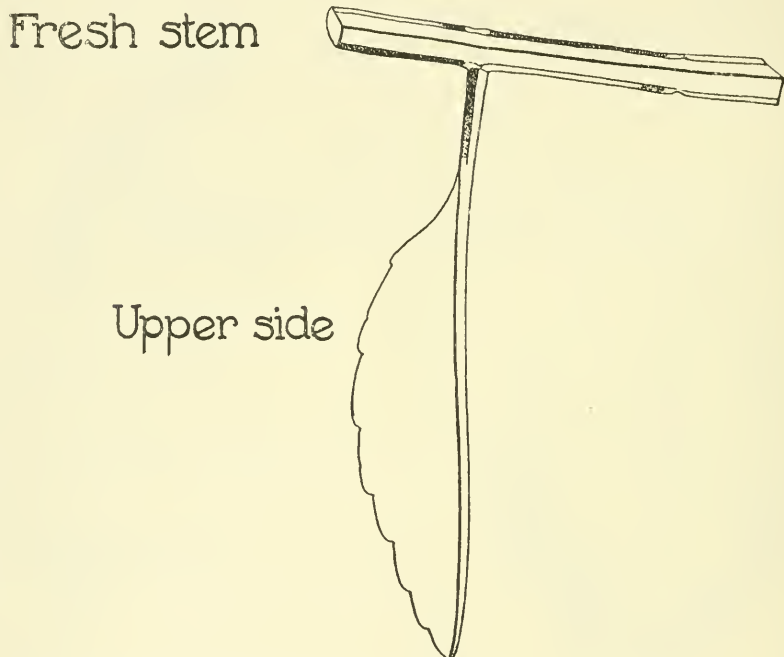


FIG. 9. Showing the distribution of the purple pigment in the stem.

it is found in the petiole, in the stem, and in the new shoots which are formed by the stem. This is illustrated by Fig. 8. The leaf on the right was left in connection with a piece of stem, while the sister leaf (to the left in Fig. 8) was detached from the stem. The latter shows a collection of the pigment in the vessels near each notch from which roots and shoots develop and the new shoots are full of the pigment. The sister leaf in connection with a piece of stem (to the right in

² Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 297.

Fig. 8) has no pigment in its notches, but the pigment is visible in the stem and especially in the small leaves formed on the upper side of the stem. It is needless to say that while only a few drawings are given the experiments were made on a large number of specimens, all yielding the same result. Fig. 9 shows the distribution of the pigment in the cortical layer of a piece of stem connected with a leaf.

While the purple pigment is a convenient indicator for the distribution of sap, it is not the cause of the growth of roots and shoots in a leaf. This is obvious from the fact that growth of roots and shoots takes place in isolated leaves when kept in the dark or when dipping in water, although no noticeable formation of the purple pigment takes place under these conditions.

SUMMARY AND CONCLUSIONS.

1. When leaves of *Bryophyllum calycinum* are suspended in moist air in a vertical plane and sidewise, roots and shoots are formed exclusively or predominate in the notches on the lower side of the leaves. When pieces of stems of the same plant are suspended horizontally in moist air, roots develop on the lower side of the stem, with the exception of the extreme basal end where they may develop on both sides.

2. The writer has suggested in a preceding paper that this directive influence of gravity on the arrangement of the regenerating organs may be due to the combination of two factors. The first factor is gravity, which causes a slightly greater collection of sap on the lower side of these organs, and as a consequence roots develop a little more quickly on the lower than on the upper side. The second factor is of an inhibitory character inasmuch as quite generally organs which grow out first, or which grow quickly, have a tendency to retard or inhibit the growth of similar organs in other places.

3. The writer was able to prove the action of this inhibitory factor by cutting off the lower edges of leaves suspended sidewise in a vertical plane or the lower halves of stems suspended in a horizontal plane (in moist air). In this case roots developed as abundantly on the upper side of these organs as they otherwise would have developed on the lower side.

4. It was, however, still necessary to prove the idea that gravity causes sap to collect in larger quantity in the lower parts of organs. This gap is filled by the present paper in which it is shown, first, that in the leaves suspended in moist air a red pigment is formed which has a tendency to collect gradually in the lowest parts of the leaf when the latter is suspended in a vertical plane. This red pigment serves as an indicator for the distribution of sap in the leaf and thus shows directly the tendency of the sap to collect in greater abundance on the lower edge of a leaf suspended in a vertical plane.

Second, it is shown that when leaves or stems of *Bryophyllum* are suspended, in the way described, under water instead of in moist air, roots develop on the upper side as well as on the lower side. The directive effect of gravity upon the arrangement of organs disappears in this case since the abundance of the outside water makes the effect of a slight difference in the distribution of sap between the upper and lower side a negligible factor.

Third, it is shown that the dry weight of the lower half of leaves suspended sidewise for several weeks in moist air in a vertical plane is greater than that of the upper half when roots and shoots are formed on the lower side only. This indicates that material from the upper half flows into the growing organs of the lower half. No such difference between upper and lower half of leaf is found when the leaves are suspended in the same way in water and roots and shoots are formed on both sides of the leaf.

5. It is shown that when a leaf connected with a piece of stem is suspended in moist air the red pigment goes into the stem instead of collecting in the lower part of the leaf, thus supporting the view expressed in a preceding paper that the inhibitory action of the stem on the root and shoot formation in a leaf of *Bryophyllum* is due to the fact that the material available in the leaf for organ formation is naturally sent into the stem.

ON THE CAUSE OF THE INFLUENCE OF IONS ON THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES. I.

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(Received for publication, January 23, 1920.)

I.

When we separate a watery solution from pure water by a collodion membrane water will diffuse into the solution and solute will diffuse out. We will call this diffusion free osmosis to distinguish it from osmosis due to an outside force; *e.g.*, electrical endosmose. The free diffusion of solute into pure water occurs at a rate proportional to the concentration of the solution¹ (with the possible exception of very low or very high concentrations) and need not occupy our interest in this paper. The diffusion of water into the solution has a different character when the solute is a non-electrolyte than when it is an electrolyte. When the solute is a non-electrolyte, the initial rate of diffusion of water into the solution is (within the limit of moderate concentrations) practically a linear function of the concentration of the solute, as it should be according to the law of van't Hoff. When the solution is an electrolyte, anomalies occur which are a characteristic function of the oppositely charged ions of the electrolyte and these anomalies were described for collodion membranes in a series of papers which have appeared recently.^{1, 2, 3, 4} The anomalies seem to occur only in the lower concentrations of electrolytes, below $M/8$ or less; above these values the osmosis seems to occur in a way similar to that observed in solutions of non-electrolytes, though this point

¹ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 717.

² Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 173.

³ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 255.

⁴ Loeb, J., *Proc. Nat. Acad. Sc.*, 1919, v, 440.

needs further investigation. The present paper deals only with concentrations inside the anomalous range.

It was shown in the preceding papers that all the anomalies can be adequately described if we assume that the water in diffusing through the pores or interstices of the membrane is either positively or negatively charged and that the ions of the solution accelerate or retard the diffusion of the electrified water by their electrical charges. When we use collodion membranes which have been bathed for a short time in a 1 per cent solution of a protein (gelatin, casein, egg albumin, edestin, etc.) the effects of the two oppositely charged ions can be expressed in the following terms.

1. When we separate a neutral, alkaline, or faintly acid solution of an electrolyte with a monovalent or bivalent cation by a collodion membrane (treated with protein) from pure water, the latter diffuses into the solution as if its particles were positively charged and as if they were attracted by the anion and repelled by the cation of the solution with a force increasing with the valency of the ion and with a second constitutional quantity of the ion which we designated arbitrarily as the radius of the ion, but which needs another definition so as to include the strong effects of such monovalent ions as; *e.g.*, the aluminate or oleate anion.

2. When we separate solutions of electrolytes with a concentration of hydrogen ions of about 10^{-4} N or above, or with trivalent or tetravalent cations in sufficient concentration by a collodion membrane from pure water, water diffuses into the solution as if its particles were negatively charged and attracted by the cation and repelled by the anion of the electrolyte with a force increasing with the valency of the ion and with a second constitutional quantity of the ions still to be defined.

When we use collodion membranes *not* treated with protein one rule suffices to express all the phenomena; namely, water diffuses through the collodion membrane into the solution as if its particles were positively charged and as if it were attracted by the anion and repelled by the cation of the solution with a force increasing with the valency and a second quantity of the ion which is still to be defined.

What we designated in this statement as electrified particles of water is the watery phase or the mobile stratum of the electrical double layer (in the sense of Helmholtz) formed at the boundary of membrane and water or solution. The other stratum of this double layer, the membrane phase, must be considered as immobile in the experiments on osmosis. The influence of electrolytes on the double layer at the boundary of water and membrane will be assumed to be responsible for the phenomena of abnormal osmosis.

It follows from our previous publications that we must discriminate between two effects of electrolytes on the double layer. The one effect is the influence of certain ions on the sign of the electrification of the water or the solution phase of the double layer. Water in contact with a membrane is generally positively charged and only in the case of certain types of membranes, *e.g.* collodion membranes treated with proteins, can the sign of the charge be reversed by two kinds of ions; namely (*a*) hydrogen ions, or (*b*) simple (*i.e.* non-complex) cations whose valency is three or more.³ All electrolytes which can lower the hydrogen ion concentration by a chemical action, *e.g.* alkalies, can restore the original positive electrification of the water stratum of the double layer. Some authors have drawn from this the conclusion that the OH ions act as directly as the H ions upon the sign of the electrification of the membrane (*e.g.* by transferring their negative charge to it). It seems to the writer that it is more in harmony with the facts to assume that the alkalies act merely by the diminution of the hydrogen ion concentration through neutralization of acid. The special effect of the two classes of positive ions—H ions and simple cations with a valency of three or above—on the sign of the electrification of the water stratum of the double layer will be discussed in another paper.

In addition to this *specific* influence of certain ions on the sign of the electrification of water at the boundary of the membrane there exists a second, more *general* effect of electrolytes on the *rate* of diffusion of water which was described in terms of electrostatic attraction and repulsion of the electrified stratum of water by the ions of the electrolyte in the previous papers, since this way of describing the results had the advantage of simplifying the presentation of the facts. It was not, however, intended to serve as a theoretical

basis for the explanation of the phenomena of abnormal osmosis. For this latter purpose we must express the facts in the following form. When we separate a solution of an electrolyte from pure water by a collodion membrane the oppositely charged ions of the electrolyte influence the initial velocity of diffusion of water through the membrane into the solution in an opposite sense; the ion with the opposite sign of charge from that of the electrified water (or the watery phase of the double layer) increasing the velocity, the ion with the same sign of charge as the watery phase of the double layer diminishing the velocity. The accelerating and retarding effects of ions were found to increase with the valency and with that other constitutional quantity which was designated as the radius of the ion but which requires further definition.

In passing we may remark that the relative retarding and accelerating effect of oppositely charged ions of an electrolyte on the rate of osmosis of water into the solution was not found to be the same for all concentrations of a solution.² At the lowest concentrations the effect of that ion usually (and possibly always) prevails which has the opposite sign of charge from that of the watery phase of the double layer, at a higher concentration the effect of that ion prevails which has the same sign of charge as the watery phase. Hence in the lower concentrations the accelerating effect of the electrolyte prevails over the retarding effect and for the higher concentrations the reverse is true. For a number of solutions, *e.g.* salts of monovalent cations, the turning point lies at a concentration of about $M/256$. The anomalous osmosis ceases at that concentration of the solution where the retarding and accelerating effects of the oppositely charged ions become equal. From then on the solutions of electrolytes seem to behave like those of non-electrolytes. This group of facts has been described in a preceding paper² and will not be discussed here.

In our experiments on free osmosis the collodion membrane was bounded on one side by pure water and on the other by the solution. When both sides of the membrane are bounded by identical solutions the rate of diffusion of water and of electrolyte in opposite directions is equal, and no change occurs. When, however, an external difference of potential is produced on the two sides of the

membrane, a transport of water or of liquid occurs through the membrane towards that electrode whose sign of charge is the opposite of that of the watery phase of the double layer in the pores or interstices of the membrane. This is the well known phenomenon of electrical endosmose which was first investigated experimentally by Quincke and Wiedemann and which was explained mathematically by Helmholtz. The earlier workers found that the watery phase of the double layer was generally positively electrified. Perrin⁵ made the remarkable discovery that in the case of certain diaphragms, such as powdered charcoal, carborundum, gelatin, etc., the sign of charge can be reversed at will, chiefly with the aid of acid and of alkali. In a slightly acid medium the liquid moves to the anode, in a slightly alkaline medium it moves to the cathode. This has been confirmed by every observer, and his deductions have been generally accepted.

It has been suggested by Girard, Bernstein, Bartell and Hocker, and Freundlich⁶ that the cases of so called negative osmosis where liquid diffuses from acid into pure water instead of in the opposite direction might be in reality manifestations of electrical endosmose. The only difference between the case of free osmosis and electrical endosmose being, according to these authors, the source of the potential difference, which is an external one in the case of electrical endosmose and an internal one—*e.g.* a diffusion or a boundary potential—in the case of free osmosis. But this is thus far merely an hypothesis which is not yet adequately supported by facts.

The possibility of correlating the phenomena of free and electrical osmosis meets at present with a difficulty. Our experiments on collodion membranes leave no doubt that in the case of *free osmosis* the influence of electrolytes on the velocity of diffusion of water from pure solvent to solution through the collodion membrane is an addi-

⁵ Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50.

⁶ Girard, P., *Compt. rend. Acad.*, 1908, cxlvi, 927; 1909, cxlviii, 1047, 1186; 1910, cl, 1446; 1911, cliii, 401; La pression osmotique et le mécanisme de l'osmose, Publications de la Société de Chimie-physique, Paris, 1912. Bernstein, J., *Elektrobiologie*, Braunschweig, 1912. Bartell, F. E., *J. Am. Chem. Soc.*, 1914, xxxvi, 646. Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1036. Freundlich, H., *Kolloid-Z.*, 1916, xviii, 11.

tive effect of the two oppositely charged ions. Perrin,⁵ however, states that in the case of electrical endosmose only one of the oppositely charged ions of an electrolyte influences the transport of liquid through the membrane; namely, the one with the same sign of charge as that of the water (or with the opposite sign of charge from that of the membrane).

II.

Perrin's^{5,7} view of the influence of electrolytes on the amount of liquid transported in electrical endosmose rests on the assumption that the sign of the electrification of the double layer is primarily determined by the H and OH ions. He assumes that the positive electrification of a membrane bounded by liquid containing a monovalent acid is due to the adsorption of a layer of hydrogen ions by the membrane. This membrane layer of adsorbed hydrogen ions is the fixed stratum of the double layer and the next stratum of the liquid—the watery phase—contains a corresponding excess of negative ions. The stratum of negative ions is sufficiently far removed from the fixed layer so as to be able to undergo the tangential displacement on which the phenomena of electrical osmosis are supposed to depend.

Perrin assumes that the negative electrification of a membrane under the influence of a monovalent base is due to the OH ions situated in that stratum of the liquid which is in immediate contact with the membrane, while a corresponding excess of positive ions exists in the opposite stratum of liquid (the watery phase, in our terminology). When another electrolyte is added to a weak acid or weak alkaline solution the charge of the membrane is, according to Perrin, *influenced only by one of the two ions* of the electrolyte added; namely, the one with the opposite sign of charge from that of the membrane.

“When a liquid electrifies a membrane with a certain sign the addition to this liquid of a polyvalent ion of the same sign does not increase the electrification, while the addition of a polyvalent ion of the opposite sign diminishes this electrification considerably. The influence of bivalent ions is inferior to that of trivalent ions and that of trivalent ions is inferior to that of tetravalent ions. In the case of the ions with high valencies the diminution of the charge can result in the complete reversal of the sign of the charge.”

⁷ Perrin, J., Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918, 36–37.

His theory which has revolutionized colloid chemistry is expressed in the following statement.

"The primary factor of this electrification is always the action of the hydrogen or the hydroxyl ions, which are pressed against the membrane in the same way, no matter whether polyvalent ions are present or absent. But if polyvalent ions of the opposite sign are present they are attracted towards the membrane. To be more precise, let us suppose a liquid with a monovalent acid; hydrogen ions cover the membrane with a positive charge according to the degree of acidity. Behind them, at a distance which results from an equilibrium between the osmotic and the electric forces are found the monovalent negative ions forming the second stratum of the double layer. If we now add negative polyvalent ions, *e.g.* $\text{Fe}(\text{CN})_6$, the osmotic forces acting on the tetravalent $\text{Fe}(\text{CN})_6$ remain of the same order as before while the electric force is multiplied by four; the density of the double layer will therefore diminish and as a consequence the P.D. of contact and the amount of electrical endosmose. It only remains to explain in a precise manner reversion of the sign of charge due to the presence of the necessary amount of $\text{Fe}(\text{CN})_6$ ions."⁷

Perrin's view on the effect of electrolytes on the double layer does not agree with our experiments with collodion membranes which show unequivocally that the influence of electrolytes on the rate of diffusion of water in the case of free osmosis is an additive effect of the two oppositely charged ions of an electrolyte, and not the effect of only one of the two ions.

If we assume that the influence of ions is the same in the case of free osmosis and in electrical endosmose an increase in the valency of the anion, according to Perrin, should not increase the rate of diffusion of positively electrified water in free osmosis, since in this case the membrane has the same sign of charge as the anion. A glance at Fig. 1 shows, however, that when we separate a solution from pure water by a collodion membrane the initial rate of diffusion of water into the solution increases in the lower concentrations of different potassium salts with increasing valency of the anion when the water is positively charged. In this case a watery solution of one of these salts was put into a collodion bag connected with a manometer and the bag was put into a beaker containing distilled water. The solutions of NaCl , CaCl_2 , Na_2SO_4 , and $\text{Na}_4\text{Fe}(\text{CN})_6$ were rendered alkaline by dissolving the neutral salt in $\text{M}/1,024$ or $\text{M}/1,000$ KOH . The abscissæ are the logarithms of the concentration and the ordinates are the rise in the level of solution in the manometer after 20 minutes.

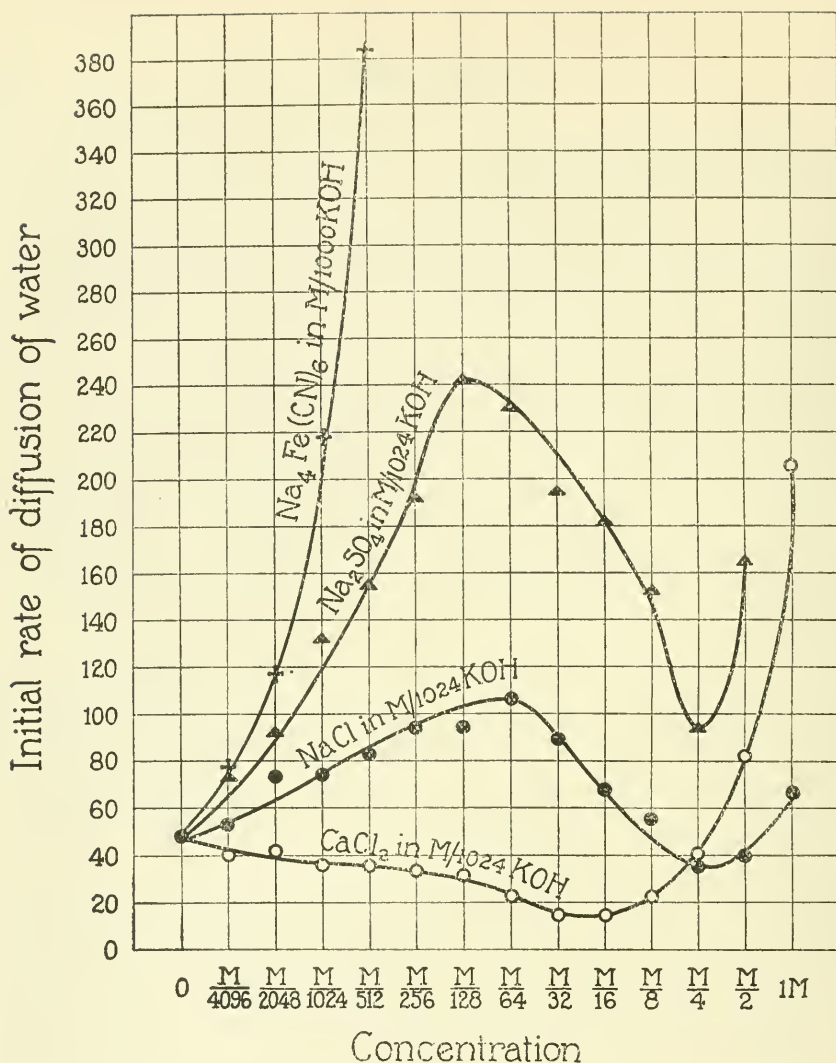


FIG. 1. Initial rate of diffusion of pure water through a collodion membrane into a solution containing an electrolyte. Abscissæ are the logarithms of concentration, ordinates rise of level of water in manometer connected with solution after 20 minutes. Solutions of salts were all rendered alkaline by enough KOH to make the solution about $10^{-3}N$ in regard to KOH. The water was positively electrified. The curves show that the initial rate of diffusion of water into the solution increases with increasing valency of the anion of the electrolyte, though the membrane has the same sign of charge as the anion. The cations have a depressing effect, increasing also with the valency. The drop in the curve beyond a concentration of $M/128$ or $M/64$ is due to the fact that beyond this concentration the effect of the cation begins to prevail over that of the anion, until at a concentration of $M/8$ or $M/4$ the gas pressure effect of the electrolyte begins to prevail over the electrical effect.^{2, 3, 8}

⁸ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 273.

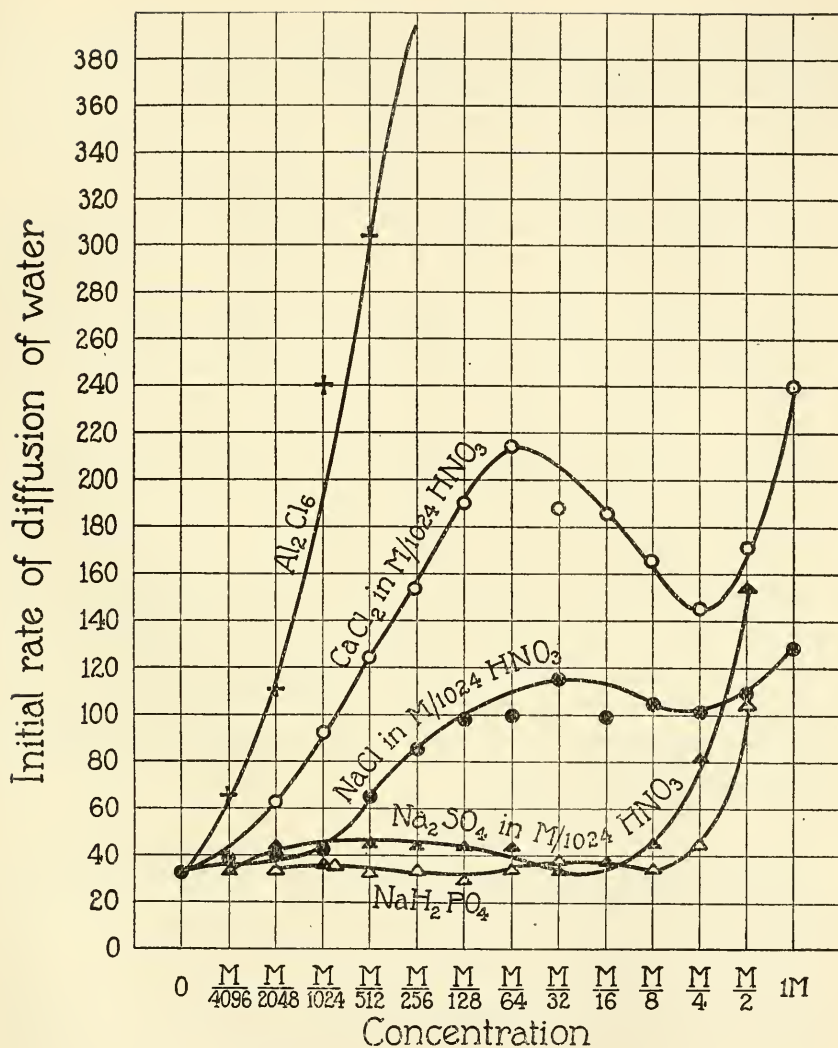


FIG. 2. Initial rate of diffusion of water from pure water through collodion membrane to solution of electrolytes rendered about 10^{-3} N acid through addition of HNO_3 . Though the water is negatively (and the membrane positively) electrified the initial rate of diffusion of water into the solution increases with increasing valency of the cation and diminishes with increasing valency of the anion. The drop in the curves in concentrations beyond $\text{M}/64$ or $\text{M}/32$ is due to the fact that in concentrations higher than these the depressing effect of the anion prevails over the opposite effect of the cation. For explanation of second rise of curve see legend of Fig. 1.

Moreover, according to Perrin, an increase in the valency of the cation should not increase the rate of diffusion of water from pure water into a slightly acid solution of salts, yet a glance at Fig. 2 shows that the initial rate of diffusion of water through a collodion membrane into a slightly acid solution increases considerably with the increase in the valency of the cation of the salt added.

In former papers,^{1, 2, 3} further proof of these statements can be found and in addition the writer has shown that in neutral solutions (where the water diffusing through the collodion membrane is positively electrified) the rate of diffusion of water into the solution increases with the valency of the anion of the electrolyte in the solution.

This discrepancy between the actual observations concerning the influence of electrolytes on the rate of diffusion of water through a collodion membrane in free osmosis on the one hand, and the theory of Perrin concerning the influence of electrolytes on the rate of diffusion of liquid in the case of electrical endosmose on the other indicates that either the influence of electrolytes is not the same in both cases or that the theory of Perrin is not the correct expression of the facts in the case of electrical endosmose, at least for collodion membranes. It seemed, therefore, necessary to test the influence of electrolytes on the rate of transport of water through collodion membranes by electrical endosmose as a first step towards a theory of the influence of electrolytes on free osmosis.

III.

In the experiments on electrical endosmose we used the collodion bags which served for the experiments on free osmosis. These collodion bags were cast inside an Erlenmeyer flask of a volume of about 50 cc. The collodion flask was closed with a rubber stopper which was perforated by a glass rod serving as a manometer. The bag was filled at the beginning of the experiment with the solution whose influence on the osmotic transport was to be investigated and was put into a beaker containing the identical solution. The bag was then so adjusted that the upper level of the rubber stopper was at the surface of the liquid in the beaker and that a column of liquid of about 30 mm. in the manometer was above the level of the liquid in the beaker. The manometer was a glass tube with a bore of about 2

mm. in diameter. One platinum electrode was put into this glass tube and one into the beaker. The distance between the electrodes was approximately the same in all experiments; namely, 6.7 cm. The electrode in the manometer was usually that pole towards which the transport of liquid occurred so that the rise of level in the manometer could serve as a measure for the volume of liquid transported. In this case the transport occurred against a hydrostatic pressure and it was necessary to compare the rise of level at about the same pressure head in different experiments.

The volume of liquid transported is a function of the external potential difference which was either 50 or 40 volts in our experiments. It turned out that in these experiments a disturbing variable entered inasmuch as with constant voltage the intensity of the current rose slowly and with the rise in intensity the amount of liquid flowing to one of the poles also rose gradually. The writer is inclined to interpret this gradual increase in intensity of current as being due to a gradual increase in the number of interstices through which the current can flow; and this means also an increase in the number of capillary spaces through which electrical endosmose can occur. Hence, for measurements of the transport of liquid only those changes in level could be used which occurred after the current had become approximately constant. In order to accelerate this process at the beginning of each experiment, a P.D. of 200 volts was used for 2 minutes or less until the intensity of the current was sufficiently high (above 1.0 or 2.0 milliamperes), and then the P.D. was lowered to the 40 or 50 volts desired. The voltage was then kept constant. As a measure of the effect of an electrolyte on the volume of liquid transported we used the rise in the level of liquid in the glass tube during the first 15 minutes after the current had become fairly constant. The following records will illustrate the way our figures were obtained. We omit the preliminary short treatment of the solution with a current of 200 volts, and give only the records for the 50 volt effects. The solutions used were M/512 KCl, M/512 K₂SO₄, M/512 and M/1,024 K₄Fe(CN)₆, M/512 CaCl₂, and M/512 BaCl₂. The solutions were almost neutral but slightly on the acid side of neutrality, the pH being about 6.2, and the transport of liquid occurred towards the cathode which was put into the glass tube. Table I gives the time in minutes, the in-

TABLE I.

*Transport of Liquid in Electrical Endosmose in Approximately Neutral Solutions,
pH = 6.2. 50 Volts. Cathode in Capillary.*

Solution.	Time.	Milliampere.	Rise of level of liquid in manometer.
	<i>min.</i>		<i>mm.</i>
M/512 KCl	0	1.3	+1.0
	5	1.7	+2.0
	10	1.9	+2.5
	15	2.1	+3.5
	20	2.15	+5.0
	25	2.18	+6.0
	30	2.18	+7.0
M/512 K ₂ SO ₄	0	1.0	+1.0
	5	1.4	+1.5
	10	1.9	+2.0
	15	2.4	+4.0
	20	2.3	+6.0
	25	2.3	+8.0
	30	2.25	+10.5
	35	2.25	+12.0
	40	2.26	+14.0
M/1,024 K ₄ Fe(CN) ₆	45	2.2	+16.0
	0	1.0	+1.5
	5	1.45	+7.5
	10	2.0	+16.0
	15	2.35	+26.0
	20	2.45	+36.0
	25	2.45	+43.0
	30	2.6	+48.0
	35	2.7	+54.0
M/512 K ₄ Fe(CN) ₆	40	2.76	+60.0
	0	1.4	+1.0
	5	2.4	+6.5
	10	3.4	+14.0
	15	3.85	+25.0
	20	4.0	+33.0
	25	4.2	+39.0
	30	4.2	+45.0
	35	4.2	+52.0
	40	4.4	+59.0

TABLE I—*Concluded.*

Solution.	Time.	Milliampere.	Rise of level of liquid in manometer.
	<i>min.</i>		<i>mm.</i>
M/512 CaCl ₂	0	1.8	+3.0
	5	1.8	+3.0
	10	1.7	+3.0
	15	1.55	+2.0
	20	1.5	+1.5
	25	1.5	0
	30	1.5	-1.0
M/512 BaCl ₂	0	2.3	0
	5	1.6	-1.5
	10	1.2	-3.0
	15	1.15	-4.5
	20	1.15	-5.5
	25	1.1	-6.5
	30	1.15	-7.5

tensity of the current in milliamperes, and the rise in the level of the liquid in the glass tube.

In the case of M/512 KCl the current became approximately constant after 15 minutes (2.1 to 2.2 milliamperes) and the rise in the level of liquid in the glass tube between 15 and 30 minutes, *i.e.* 7 — 3.5 mm. = 3.5 mm., was used as a measure for the relative influence of M/512 KCl (approximately neutral) upon the rate of endosmotic transport of liquid.

The figures for transport during 15 minutes were selected as follows: for M/512 K₂SO₄ = 10.5 — 4 = 6.5; for M/512 K₄Fe(CN)₆ = 45 — 25 = 20; etc.

Since in the case of M/512 K₄Fe(CN)₆ the intensity of the current was greater than in the experiments with KCl or K₂SO₄, an experiment with M/1,024 K₄Fe(CN)₆ is added in the table. It gave 48 — 26 = 22 mm. as the transport number though the intensity of current was almost as low as in the case of M/512 K₂SO₄ or M/512 KCl.

Experiments with neutral solutions of salts with bivalent cation, like CaCl₂, BaCl₂, gave no transport with electrical endosmose even with 100 volts and an intensity of current of 4.8 milliamperes. Table II gives the transport numbers for various approximately neutral solutions.

Table II shows that the rate of endosmotic transport increases in almost neutral solutions of salts with increasing valency of the anion and diminishes with increasing valency of the cation. Electrolytes influence, therefore, the osmotic transport in exactly the same sense in free and in electrical osmosis. This influence is in both cases an additive effect of the oppositely charged ions of the electrolyte. In the presence of the salt mentioned the watery phase of the double layer is positively charged. The slight fall of level in the case of CaCl_2 and of BaCl_2 must be ascribed to the pressure head of about 30 mm. solution existing at the beginning of the experiment; since the same or a slightly more rapid fall of level occurs if no current passes through the liquid.

TABLE II.

Relative Transport of Liquid by Electrical Endosmose in Approximately Neutral Solutions, $\text{pH} = 6.2$. 50 Volts.

	Rise of level of liquid in manometer in 15 min.
	<i>mm.</i>
M/512 KCl.....	3.5
M/512 K_2SO_4	6.5
M/512 $\text{K}_4\text{Fe}(\text{CN})_6$	20.0
M/1,024 $\text{K}_4\text{Fe}(\text{CN})_6$	22.0
M/512 CaCl_2	0
M/512 BaCl_2	0

We will now show that the statements made for neutral solutions are also true for alkaline solutions. In alkaline solutions the watery phase of the double layer is also positively charged and the liquid is transported to the cathode as in the case of neutral solutions. The cathode was put into the glass tube and the rise of level in the glass tube during the first 15 minutes after the current had become fairly constant was used as a measure for the transport. The solutions were brought to the same alkalinity as that of M/1,000 KOH and the pH varied between 10.9 and 11.0 (Table III).

The result is the same as before: the rate of endosmotic transport increases in alkaline solutions with increasing valency of the anion and diminishes with increasing valency of the cation. Electrolytes

influence, therefore, the transport of liquid in alkaline solution in the same sense in electrical endosmose as in free osmosis. This influence is in both cases an additive effect of the oppositely charged ions of the electrolyte. In the alkaline solutions the watery phase of the double layer is positively charged as it is in neutral solutions. If Perrin's rule applied to these experiments, the increasing valency of the anion should have had no effect.

Table III contains also the transport numbers of solutions of Na acetate, Na aluminate, and K oleate which are all higher than those of NaCl, although the anion is monovalent in each case. Solutions of

TABLE III.

Transport of Liquid by Electrical Endosmose to the Cathode in Alkaline Solutions, pH = 10.9 to 11.0. 40 Volts.

	Milliampere.	Rise of level of liquid in manometer in 15 min.
		mm.
M/512 NaCl.....	1.1	3.5
M/512 Na ₂ SO ₄	1.6	10.0
M/512 Na ₄ Fe(CN) ₆	2.1	22.0
M/512 CaCl ₂	1.2	0
M/512 BaCl ₂	1.15	0
M/512 Na acetate.....	1.35	7.0
M/512 NaAlO ₂	1.15	7.0
M/512 K oleate (pH = 9.4).....	1.15	22.5

these salts also attract water more powerfully than solutions of NaCl in the case of free osmosis and the influence of these salts in electrical endosmose is parallel to their influence in free osmosis. These salts illustrate the statement that in addition to the valency another constitutional quantity of the ions determines their influence on the transport of liquid in free and electrical endosmose.

We finally investigated the electrical transport of liquid in acid solutions. The salt solutions were made N/1,000 acid by the addition of HNO₃; the pH was in all cases exactly 3.0. Table IV gives the results. The anode was in the glass tube. In this case it was necessary to use membranes which had received a gelatin treatment.

If Perrin's rule applied to these cases, the increasing valency of the cation should not have influenced the result in these acid solutions.

We notice, however, that the rate of endosmotic transport to the anode increases in acid solutions with the increase in the valency of the cation and diminishes with the increase in the valency of the anion. Electrolytes influence, therefore, the transport of liquid in acid solutions in the same sense in the case of free and of electrical osmosis. The influence is in both cases an additive effect of the oppositely charged ions of the electrolyte. The watery phase of the electrical double layer is negatively charged in acid solutions of the hydrogen ion concentration used in this case; namely, 10^{-3} N.

TABLE IV.

Relative Transport of Liquid in Electrical Endosmose to the Anode in Acid Solutions, pH = 3.0. 40 Volts.

	Milliampere.	Rise of level of liquid in manometer in 15 min.
		mm.
M/512 NaCl	3.7	5.5
M/512 CaCl ₂	2.6	11.5
M/512 BaCl ₂	4.0	13.0
M/512 CeCl ₃	3.5	16.5
M/512 ThCl ₄	3.8	18.0
M/512 Na ₂ SO ₄	3.4	0
M/512 Na ₂ oxalate	3.2	0
M/512 NaH ₂ PO ₄	1.8	2.0

If we summarize all three cases we may state that in both free and electrical osmosis, the transport of liquid is accelerated by that ion of an electrolyte which has the opposite sign of charge as the watery phase of the double layer (or the same sign of charge as the collodion membrane) and retarded by that ion which has the same sign of charge as the watery phase of the double layer (or the opposite sign of charge as the collodion membrane); and that both the accelerating and the retarding effect of ions increase with their valency and a second constitutional quantity of the ion which is still to be defined and for which the high transport number of several salts in Table III may serve as an example.

The writer has made a number of experiments on electrical endosmose with different concentrations of electrolytes. The curves repre-

senting this influence on the transport of liquid in electrical endosmose through collodion membranes seem to be similar to the curves, representing the influence of different concentrations of the same electrolytes on free osmosis, which were published in a preceding paper.²

In experiments with concentrations of electrolytes above $M/512$ or $M/256$ it is wiser to work with a lower voltage to avoid the excessive development of gas bubbles. Table V gives the numbers for the rise of liquid in the manometer for different concentrations of $K_4Fe(CN)_6$

TABLE V.

Influence of Concentration on Transport of Liquid by Electrical Endosmose to Cathode. 20 Volts.

	Milliampere.	Rise of level of liquid in manometer in 15 min.
		<i>mm.</i>
$M/2,048 K_4Fe(CN)_6$	0.1	1.5
$M/1,024 K_4Fe(CN)_6$	0.5	9.0
$M/512 K_4Fe(CN)_6$	0.9	10.5
$M/256 K_4Fe(CN)_6$	1.2	6.0
$M/128 K_4Fe(CN)_6$	1.8	3.0
$M/64 K_4Fe(CN)_6$	3.0	0

during 15 minutes after the intensity of the current had become fairly constant. The p.d. applied was 20 volts.

The maximum of transport of liquid in electrical endosmose was reached at a concentration of about $M/512 K_4Fe(CN)_6$ and then the electro-endosmotic transport fell rapidly to zero with increasing concentration, although the intensity of the current increased with concentration. The drop in the curves representing the initial rate of diffusion of water from pure water to solution through collodion membranes in the case of free osmosis is therefore paralleled in the case of electrical endosmose (Table V). A fuller account of these results will shortly be published.

Theoretical Remarks.

According to the formula of Helmholtz for the transport of liquid by a current through capillaries, modified by Perrin,⁹ we have

$$v = \frac{q \cdot \epsilon \cdot E \cdot D}{4 \pi \cdot \eta \cdot l}$$

where v is the quantity of liquid carried electro-osmotically, ϵ is the potential difference between the two strata of the double layer, E the external electromotive force, D the dielectric constant of the medium, η the coefficient of internal friction, and l the distance of the external electrodes. Since in our experiments all quantities occurring in this formula except v and ϵ were kept approximately constant, we must attribute the influence of electrolytes on the quantity of transport v to an influence of the ions on ϵ . We must therefore conclude that the influence of electrolytes on the rate of free osmosis is due to the effect of the ions of the electrolyte on the quantity of charge on the unit area of the Helmholtzian double layer. Our experiments on both free and electrical osmosis show that this influence is an additive effect of the two oppositely charged ions of the electrolyte at least in the case of collodion membranes. Since the quantity of transport v increases with the value of ϵ we must further conclude that the ion with the same sign of charge as the watery phase of the double layer diminishes the value of ϵ since this ion diminishes transport in both free and in electrical osmosis; while the ion with the opposite sign of charge as this watery phase increases the value of ϵ in both forms of osmosis. Both effects increase with the valency and with the second constitutional quantity of the ion (Table III). The total effect of the two oppositely charged ions of an electrolyte on the rate of diffusion of water through a collodion membrane is therefore the difference between the opposite effects of its ions on the value of ϵ . These statements give the theoretical basis of what we called in our former papers the apparent electrostatic action of the ions on the rate of diffusion of the electrified particles of water from pure water into solution through a collodion membrane (free osmosis).

⁹ Freundlich, H., *Kapillarchemie*, Leipsic, 1909, 226.

Positively charged particles of water in the pores or interstices of the membrane will be driven to that side of the membrane which is more negatively charged. Since this is usually the solution side, water will be driven from the side of pure water into the solution.

When the collodion membrane has been treated with a protein, it is also generally negatively charged when bounded by water except when the solution contains hydrogen ions or simple trivalent or tetravalent cations beyond a certain concentration (which for H is 10^{-4} N); in this case the membrane is positively and the watery phase is negatively charged. If we add in this case an electrolyte to the water, the charge on the membrane is increased by the cations and diminished by the anions of the electrolyte. Whenever the positive charge on the solution side of the membrane is greater than on the opposite side, the negatively charged particles of water will diffuse from the side of pure water to the side of solution.

When the charge on the solution side of the membrane is diminished by the electrolyte so that the charge is smaller than on the side of pure water, the liquid will flow through the membrane from solution side to the side of pure water (negative osmosis).

Our experiments were made with collodion membranes only and it is possible that Perrin's statement holds for other types of membranes. It seems, however, that in the case of the influence of electrolytes on the value of ϵ at the boundary of oil drops and water the effect is also an additive one of the oppositely charged ions. Powis¹⁰ has measured this value from the velocity of the motion of oil drops through solutions on the basis of the Helmholtz-Perrin formula for five electrolytes, KCl, BaCl₂, AlCl₃, ThCl₄, and K₄Fe(CN)₆. The oil particles are negatively charged and their charge is increased more by K₄Fe(CN)₆ than by KCl. The charge is diminished with the increasing valency of the cation. This indicates that the influence of electrolytes on the value of the potential difference of the double layer is in this case also an additive effect of the two kinds of ions.

We have stated that in the case of free osmosis the rate of diffusion of water from pure solvent to solution through a collodion membrane increases at first with increasing concentration, reaches a maximum

¹⁰ Powis, F., *Z. physik. Chem.*, 1915, lxxxix, 91.

(which for many electrolytes lies at a concentration of about $M/256$) and then drops again with a further increase in concentration. It seems from the writer's experiments that the same phenomenon occurs in the case of electrical endosmose through collodion membranes and that the turning point lies near $M/512$. Powis reports a similar effect of concentration in his observation on the motion of oil drops in an electrical field, and recently published experiments of Kruyt¹¹ on "current potentials" demonstrate the same phenomenon.

It seems to follow from this that the density of the electrical double layer at the boundary of watery phase and membrane increases at first with increasing concentration of an electrolyte up to a certain point which for a number of electrolytes seems to lie at about $M/512$. If the concentration of the electrolyte rises beyond this point, the density of the charge on the double layer diminishes rapidly with a further increase in the concentration of the electrolyte.

SUMMARY.

1. In three previous publications it had been shown that electrolytes influence the rate of diffusion of pure water through a collodion membrane into a solution in three different ways, which can be understood on the assumption of an electrification of the water or the watery phase at the boundary of the membrane; namely,

(a) While the watery phase in contact with collodion is generally positively electrified, it happens that, when the membrane has received a treatment with a protein, the presence of hydrogen ions and of simple cations with a valency of three or above (beyond a certain concentration) causes the watery phase of the double layer at the boundary of membrane and solution to be negatively charged.

(b) When pure water is separated from a solution by a collodion membrane, the initial rate of diffusion of water into a solution is accelerated by the ion with the opposite sign of charge and retarded by the ion with the same sign of charge as that of the water, both effects increasing with the valency of the ion and a second constitutional quantity of the ion which is still to be defined.

¹¹ Kruyt, H. R., *Kolloid-Z.*, 1918, xxii, 81.

(c) The relative influence of the oppositely charged ions, mentioned in (b), is not the same for all concentrations of electrolytes. For lower concentrations the influence of that ion usually prevails which has the opposite sign of charge from that of the watery phase of the double layer; while in higher concentrations the influence of that ion begins to prevail which has the same sign of charge as that of the watery phase of the double layer. For a number of solutions the turning point lies at a molecular concentration of about $M/256$ or $M/512$. In concentrations of $M/8$ or above the influence of the electrical charges of ions mentioned in (b) or (c) seems to become less noticeable or to disappear entirely.

2. It is shown in this paper that in electrical endosmose through a collodion membrane the influence of electrolytes on the rate of transport of liquids is the same as in free osmosis. Since the influence of electrolytes on the rate of transport in electrical endosmose must be ascribed to their influence on the quantity of electrical charge on the unit area of the membrane, we must conclude that the same explanation holds for the influence of electrolytes on the rate of transport of water into a solution through a collodion membrane in the case of free osmosis.

3. We may, therefore, conclude, that when pure water is separated from a solution of an electrolyte by a collodion membrane, the rate of diffusion of water into the solution by free osmosis is accelerated by the ion with the opposite sign of charge as that of the watery phase of the double layer, because this ion increases the quantity of charge on the unit area on the solution side of the membrane; and that the rate of diffusion of water is retarded by the ion with the same sign of charge as that of the watery phase for the reason that this ion diminishes the charge on the solution side of the membrane. When, therefore, the ions of an electrolyte raise the charge on the unit area of the membrane on the solution side above that on the side of pure water, a flow of the oppositely charged liquid must occur through the interstices of the membrane from the side of the water to the side of the solution (positive osmosis). When, however, the ions of an electrolyte lower the charge on the unit area of the solution side of the membrane below that on the pure water side of the membrane, liquid will diffuse from the solution into the pure water (negative osmosis).

4. We must, furthermore, conclude that in lower concentrations of many electrolytes the density of electrification of the double layer increases with an increase in concentration, while in higher concentrations of the same electrolytes it decreases with an increase in concentration. The turning point lies for a number of electrolytes at a molecular concentration of about $M/512$ or $M/256$. This explains why in lower concentrations of electrolytes the rate of diffusion of water through a collodion membrane from pure water into solution rises at first rapidly with an increase in concentration while beyond a certain concentration (which in a number of electrolytes is $M/512$ or $M/256$) the rate of diffusion of water diminishes with a further increase in concentration.

THE EFFECT OF TEMPERATURE UPON FACET NUMBER IN THE BAR-EYED MUTANT OF DROSOPHILA.*

PART I.

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(Received for publication, December 30, 1919.)

INTRODUCTION.

Environment plays an important part in the development of every organism. In a few cases, it becomes a determinative agent in that particular structures are developed only under special external stimuli.

The bar-eyed mutant of *Drosophila melanogaster* Meig (*ampelophila*) shows such a response to temperature. Primarily this germinal factor produces a reduction in the number of facets in the compound eyes. Under a constant environment, it produces practically a constant effect in all individuals. Under varied temperature conditions, the amount of reduction varies inversely with the temperature.

The present study¹ is an attempt to work out these relations in detail. From the standpoint of the modern physiologist, the results are of particular interest in demonstrating in the same living material a physiological reaction whose rate is an exponential function of the temperature, while another reaction has a rate which is clearly a linear function of the temperature.

From the standpoint of experimental embryology in its broadest sense, this study gives an evaluation of some of the external and internal factors involved in the development of a particular structure,

* Contribution from the Zoological Laboratory of the University of Illinois, No. 148.

¹ This investigation was developed under the direction of Dr. Charles Zeleny. The study was made possible through the excellent equipment for environmental control installed at the Vivarium.

the compound eye. Furthermore, the particular stages in development at which the facet-determining reaction is initiated has been ascertained by the use of the temperature differences.

Materials and Methods.

The bar-eyed mutant of *Drosophila* was first described by Tice. Considerable variation was noticed in this character. Zeleny and Mattoon, and May have shown that part of this variation was due to germinal differences. It was noted, however, that environmental factors were responsible for the greater part of the variation.

A preliminary analysis of the temperature effects on facet number was made by Seyster in this laboratory in 1916.

The white, bar-eyed mutant of *Drosophila* was used. Ordinary cultural procedure was followed, except that the banana was sterilized by bringing it to the boiling point. After cooling, the banana was inoculated with a pinch of Fleischmann's compressed yeast. 4 and 8 ounce wide-mouth bottles were used. After being fitted with one-fourth sheet of Scott Tissue Towelling and a tight cotton stopper, they were subjected to 120–150°C. in a Sargent dry air electric oven.

All matings were mass matings. Unselected stock refers to Stock 127, in which Zeleny started selection in 1917. Selected Low F₄ is a stock derived from Culture 150.3 of the fourth generation of these selections; Ultra-bar is a stock derived from a 19 facet male mutant in the F₂ low generation of the same selections. These parent stocks were maintained at 27°C. The low facet stocks were used more extensively because of the greater ease in counting, and also to eliminate as many germinal differences as possible (Zeleny).

Temperature Control.—A constant temperature of 15° was maintained in the cool room. The Johnson Heat Regulation System controls the temperature by forcing air over brine coils and redistributing it to the room. A constant temperature of 23° was maintained in a similar manner in the warm room.

A battery of aquaria, fitted with Johnson regulators controlling the flow of hot and cold water, supplied the following temperatures; —16, 17.5, 20, 25, and 30°C. 27° was maintained in a Chicago Surgical and Electric Company incubator. At first, very marked differences in temperature were found in various parts of this incubator (1.5–3°). A 9 inch desk fan was installed in the top of the incubator, with the blades inside and the motor outside. This fan was run at high speed, and no further temperature differences were noticed.

Temperature Records.—Temperature records were kept at 27° by a Tycos Recording Thermometer. At 15 and 23°, New Tycos Thermographs were used. In the aquaria, Friez Soil and Water Thermographs kept the records.

The experimental material was kept as close to the effective elements of these instruments as was possible. Checks were frequently made on the instruments by standardized thermometers.

The temperature of the banana in which the larvæ were developing was tested by inserting a thermometer through the cotton plug into the food. Only very slight differences were found between the food and the surrounding medium.

The temperatures as given in the experimental data vary at most $\pm 0.5^{\circ}\text{C}$.

At the high temperatures, 29 and 31° , additional checks were made on the rate of metamorphosis and facet number by using a water thermostat. This instrument is of the type used by physical chemists. It is a battery jar supplied with a U-tube mercury regulator closing a secondary heating system by relay. A 3-inch, motor-driven fan keeps the water agitated. Variations in temperature do not exceed $\pm 0.05^{\circ}\text{C}$.

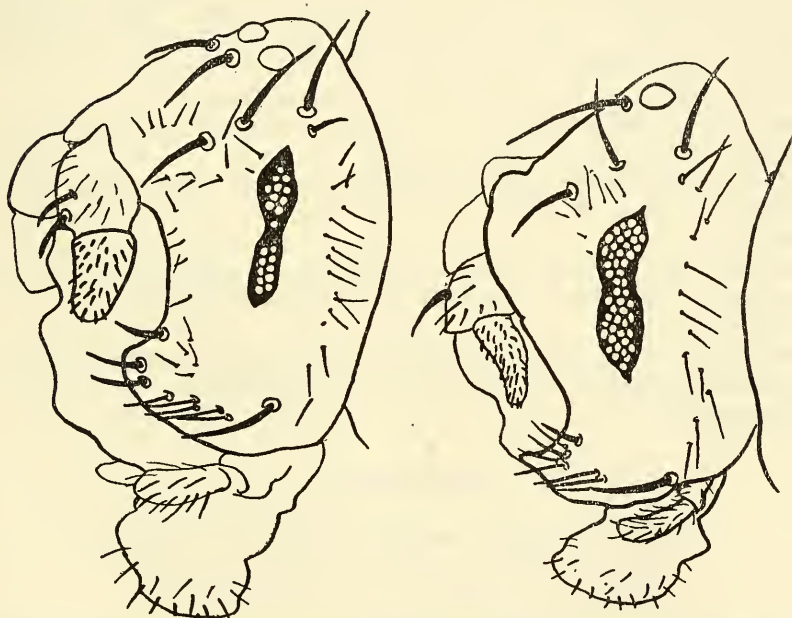


FIG. 1. Camera lucida drawings of the heads of two Ultra-bar females. The one on the left has 21 facets; reared at 27° . The one on the right has 48 facets, reared at 15° . The magnification is the same in both drawings. $\times 100$.

Technique.—Counts of the facets were made by the methods described by May. The flies were etherized and placed one at a time on a wooden counting block. The upper surface of this block was cut to a slight incline in order to bring the compound eye into the best position for focusing. The facets were counted under a No. 4 ocular and a No. 3 Leitz objective with direct illumination from a 60 watt tungsten lamp. Adjustments were made with the fine thumb-screw to accommodate the curvature of the head. Facets were recorded immediately after each individual count. The material was then preserved in alcohol.

Sources of Error.—Errors due to technique have been discussed at length by May. The chief sources are (1) errors in counting, and (2) differences in counts between the right and left eyes. The latter factor is not significant in this study. Counts were made on one eye only, but no preference was given either eye in making the counts. The first factor was reduced to a minimum by using the low facet stocks. Ultra-bar at 15° has a mean of only 60.81 facets for the males. In such stock, all the facets are readily counted as no confusion of rows arises (Fig. 1).

There is no direct correlation between the size of the fly and the number of facets (Seyster, 1919). Exceptionally large individuals can be produced under the best cultural conditions at 31° and yet show the extreme low facet number of that temperature. All the flies are of practically the same size at all temperatures. Fig. 1 shows the heads of two females, raised at 27 and 15° respectively. They are drawn to exactly the same scale.

Slight variations in the mean of the individual bottles and between separate experiments may be attributed to slight differences in acidity, food, random sampling, and other factors.

Some of the variability in the Unselected stock may be due to germinal differences in the parents. The Ultra-bar and Low Selected bar stocks are practically homogeneous.

The precaution of keeping the parent stocks at a constant temperature was unnecessary as shown by the experiments on the non-inheritance of the temperature effects.

Bearing the foregoing points in mind, we may conclude that temperature is the chief factor involved in the differences obtained in the mean facet numbers in the present study.

EXPERIMENTAL.

Temperature Effect on the Mean Facet Number.

Temperature Effect on Unselected Bar Stock.

In 1916, Seyster carried out some experiments on the bar-eyed mutant of *Drosophila* showing that facet number varied inversely with the temperature at which the flies developed.

The preliminary experiments on Unselected bar stock confirm these results. Summaries of the first experiments are given in Tables I and II. They include the temperature at which the flies developed, the number of individuals whose facets were counted, the average or mean facet number for those individuals, the difference in the means accompanying a difference of 1°C. in the environment, and the per cent of change in the mean produced by a difference of 1°C.

TABLE I.

Unselected Bar Stock. Summary of Experiments 1, 4, 7, and 16. Females.

Temperature at which flies were reared.	No. of individuals counted.	Mean facet No.	Difference in facet No. for interval.	Difference in facet No. per °C.	Average of mean facet Nos. for interval.	Per cent of change in mean per °C.
°C.						
15	90	213.67				
20	117	122.20	91.47	18.29	167.93	10.89
25	114	81.08	41.12	8.22	101.64	8.08
30	97	39.66	41.42	8.28	60.37	13.71
			Average.	11.59		10.89

TABLE II.

Unselected Bar Stock. Summary of Experiments 1, 4, 7, and 16. Males.

Temperature at which flies were reared.	No. of individuals counted.	Mean facet No.	Difference in facet No. for interval.	Difference in facet No. per °C.	Average of mean facet Nos. for interval.	Per cent of change in mean per °C.
°C.						
15	71	269.76				
20	109	160.76	109.00	21.80	215.26	10.12
25	109	120.52	40.24	8.05	140.64	5.72
30	92	73.54	46.98	9.39	97.03	9.68
			Average.	13.08		8.50

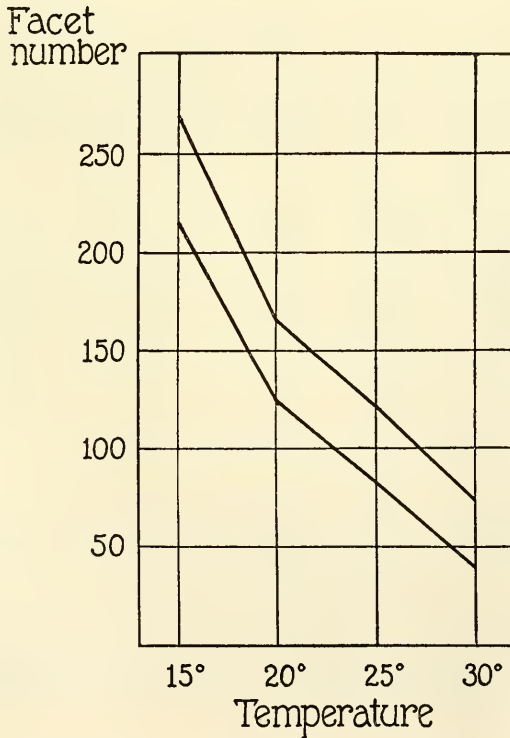


FIG. 2. Temperature effect on the mean facet number in Unselected bar stock. The upper curve is that of the males, the lower that of the females.

The difference in facet number per °C. is found by dividing the observed difference between the means of two temperatures by the number of degrees there are between those temperatures. The per cent change in the mean is found by dividing the difference in facet number per °C. by the average of the two means for the observed temperatures.

A consistent sexual difference exists in all these experiments. It is impractical to combine the data for the two sexes into a single table as the sex coefficient is not constant throughout.

The results of these experiments are shown graphically in Fig. 2.

Temperature Effect on Low Selected Bar Stock.

The preliminary experiments on Unselected bar stock showed the temperature range of the species to be practically limited to 15–30°C. From this it was decided to interpolate as many intermediate experi-

TABLE III.

Low Selected Bar Stock. Summary of Experiments 12, 22, and 27. Females.

Temperature at which flies developed.	No. of flies counted.	Mean facet No.	Difference in facet No. for interval.	Difference in facet No. per °C.	Average of means for interval.	Per cent of change for interval.
°C.						
15	79	189.00				
16	104	158.20	30.80	30.80	173.59	17.74
17.5	94	127.27	30.93	20.61	142.73	14.43
20	105	98.88	28.39	11.36	113.07	10.04
25	165	74.25	24.63	4.93	86.56	5.69
27	164	55.13	19.12	9.56	64.69	14.73
29	100	47.40	7.73	3.86	51.26	7.54
30	64	36.56	10.84	10.84	41.89	25.82
31	100	28.85	7.71	7.71	32.70	23.57
			Average.	12.38		14.95

mental temperatures as the apparatus at hand would permit. For the Low Selected bar stock the temperatures were as follows: 15, 16, 17.5, 20, 25, 27, 29, 30, and 31°C. Summaries of these experiments are included in Tables III and IV, while the results are shown graphically in Fig. 3. From these tables it is at once apparent that temperature has a very marked effect on the mean facet number of the Low Selected bar stock.

TABLE IV.

Low Selected Bar Stock. Summary of Experiments 12, 22, and 27. Males.

Temperature at which flies developed.	No. of flies counted.	Mean facet No.	Difference in facet No. for interval.	Difference in facet No. per °C.	Average of means for interval.	Per cent of change in mean per °C.
°C.						
15	74	240.21	32.66	32.66	223.88	14.58
16	107	207.55	42.89	28.59	186.10	15.36
17.5	95	164.66	41.78	16.71	143.77	11.62
20	104	122.88	19.76	3.95	113.00	3.49
25	164	103.12	23.66	11.83	91.29	12.95
27	167	79.46	13.88	6.94	72.52	9.56
29	100	65.58	7.78	7.78	61.69	12.61
30	61	57.80	16.10	16.10	49.75	32.36
31	100	41.70	Average.	15.57		14.06

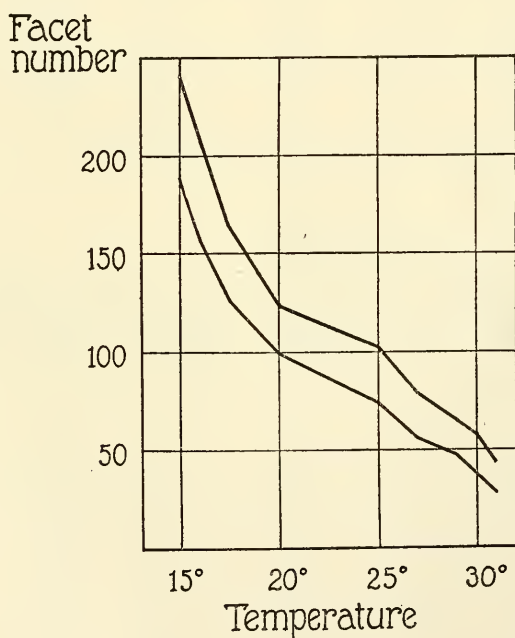


FIG. 3. Temperature effect on the facet number in Low Selected bar stock. The upper curve is that of the males, the lower that of the females.

Temperature Effect on Ultra-Bar Stock.

Experiments on this stock involve practically the same temperatures as the preceding ones. The summaries of the experiments on Ultra-bar stock are given in Tables V and VI and are shown in Fig. 4.

TABLE V.

Summary of the Effects of Temperature upon Facet Number in Ultra-Bar Females.

Temperature at which flies developed.	No. of flies counted.	Mean facet No.	Difference in facet No. for interval.	Difference in facet No. per °C.	Average of means for interval.	Per cent of change in mean per °C.
°C.						
15	55	51.51				
16	101	43.27	8.24	8.24	47.39	17.37
17.5	112	38.57	4.70	3.13	40.92	7.64
20	112	32.59	5.98	2.39	35.58	6.72
23	94	28.30	4.29	1.43	30.49	4.69
25	500	25.24	3.06	1.53	26.77	5.72
27	490	21.27	3.97	1.98	23.25	8.51
29	629	17.23	4.04	2.02	19.25	10.49
31	138	14.57	2.66	1.33	15.90	8.36
			Average.	2.76		8.58

TABLE VI.

Summary of the Effects of Temperature upon Facet Number in Ultra-Bar Males.

Temperature at which flies developed.	No. of flies counted.	Mean facet No.	Difference in facet No. for interval.	Difference in facet No. per °C.	Average of means for interval.	Per cent of change in mean per °C.
°C.						
15	65	60.81				
16	103	51.10	9.71	9.71	55.95	17.35
17.5	100	45.18	5.92	3.95	48.14	8.21
20	120	37.20	7.98	3.19	41.19	7.74
23	94	31.43	5.77	1.92	34.31	5.59
25	469	27.60	3.83	1.91	29.51	6.47
27	538	23.70	3.90	1.95	25.65	7.60
29	641	19.02	4.68	2.34	21.36	10.95
31	187	14.16	4.86	2.43	16.59	14.65
			Average.	3.42		9.82

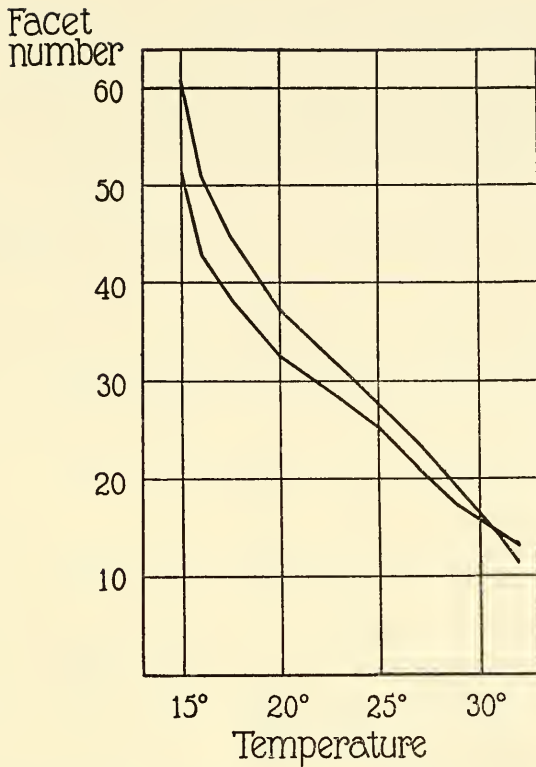


FIG. 4. Temperature effect on the mean facet number in the Ultra-bar stock. The upper curve is that of the males, the lower that of the females.

Temperature Effect on Full-Eyed Wild Stock.

In Table VII are given the counts of the full-eyed wild stocks reared at 15 and 27°.² They were made on material that was first boiled in caustic potash, cleared, and mounted in balsam. The slides were held in a mechanical stage while the facets were counted under a Zeiss No. 8 objective and a No. 12 ocular. The ocular field was broken up into rectangles by spider web cross hairs. One rectangle was counted at a time.

² The counts of the full-eyed wild stocks reared at 27° are available through the kindness of Dr. Zeleny.

The labor involved in the preparation and in the counting renders the full-eyed stock unsuitable for a more elaborate study.

The counts at hand show that temperature does not affect facet number in full-eye to any appreciable extent. One female of the two at 15° was slightly outside the range of the counts at 27°. The three flies mounted and counted were taken at random from a hatch at 15°. The remainder of the hatch was examined under the binocular. No differences were detected between these flies and the three counted

TABLE VII.

Effect of Temperature upon Facet Number in the Full-Eyed Stock.

Temperature at which flies were raised.	Experiment No.	No. of individuals.	Average facet No.	Range in facet No.
°C.				
27	398	5 females	956	886 1,082
27	345	10 “	810.6	632 924
15	51.3	2 “	1,084	912 1,256
27	345	10 males.	849.8	700 980
15	51.3	1 male.	1,016	

Temperature Effects on the Three Bar Stocks Compared.

For a direct comparison, the mean facet numbers of the females of the three stocks are shown in Fig. 5. Here they are all plotted to the same scale. The lower curve is that of the Ultra-bar stock, the middle one is that of the Low Selected bar stock, while the upper one is that of the Unselected bar stock.

In the Ultra-bar stock, the mean for the females is reduced from 51.51 facets at 15° to 21.27 at 27°. In the Low Selected, the mean is reduced from 189.00 facets at 15° to 55.13 at 27°. In the Unselected bar stock, the mean is 213.67 at 15° and 81.08 at 25°. There is a very marked reduction in facet number with increase in temperature in each of these three stocks.

The Low Selected and the Unselected bar stocks parallel each other closely in their temperature-facet relations. The Low Selected has a consistently lower mean facet value for all temperatures studied.

The Ultra-bar stock differs from the Low Selected first in the mean facet values for all temperatures, and, second, in the amount of reduction for a degree Centigrade change in temperature.

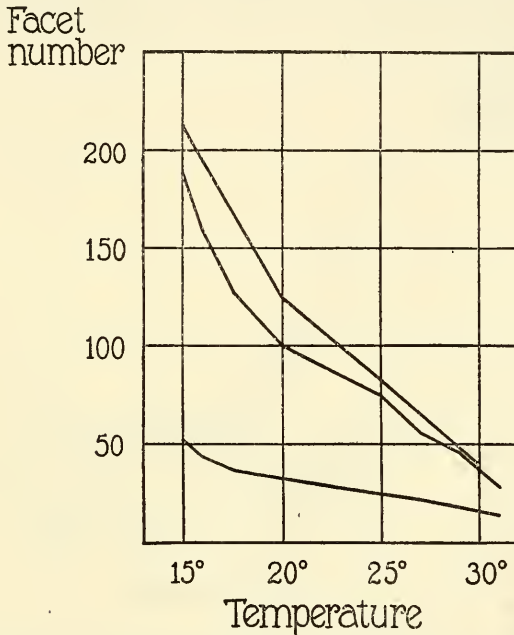


FIG. 5. Temperature effect on the three bar stocks compared. The three curves are all drawn to the same scale. The upper one is that of the Unselected bar stock females, the middle that of the Low Selected bar stock females, and the lower that of the Ultra-bar stock females.

We may draw two conclusions from these curves: (1) The mean facet number at any given temperature is not the same for all stocks. (2) The difference in the mean number of facets between any two temperatures is not a constant for all three stocks. In other words, the number of facets is determined by a specific germinal constitution plus a specific environment.

The Temperature Coefficient.

(a) *Changes in Mean Facet Number Accompanying a Unit Temperature Change.*—In the fifth rows of Tables I to VI are given the average differences in facet number per °C. for the temperature intervals indicated. These temperature differences are shown in Fig. 6.

The greatest change in facet number for a change in temperature of 1° is that for the interval 15–16° in the Low Selected males. Here 1° produces a difference of 32.66 facets in the mean values.

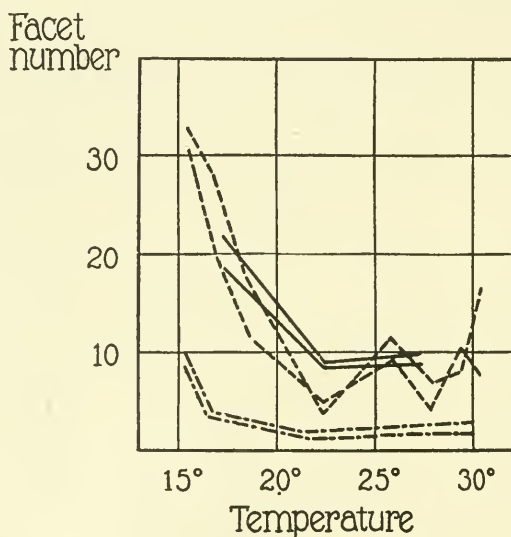


FIG. 6. Number of facets difference in the mean accompanying a difference of 1° in temperature in the environment in which the flies were raised. The solid line represents the Unselected bar stock, the broken line the Low Selected, and the dot and dash line the Ultra-bar. The upper curve of a pair is for the males in each case.

The least change for a difference of 1° in temperature is for the interval 29–31° in the Ultra-bar females. Here a difference of 1° in temperature produces only 1.33 facets difference between the two means.

Obviously a difference of 1° does not produce a constant difference in the mean number of facets. The greater differences are produced at the lower temperatures, the lesser at the higher. Again, the greater

differences are produced in the high mean stocks while the lesser are produced in the low mean stocks.

The average difference in facet number accompanying a difference of 1°C . in the environment in which the flies were reared is 3.09 for the Ultra-bar and 14.01 for the Low Selected bar stock.

(b) *Per Cent of Change in the Mean Facet Number Accompanying a Unit Change in Temperature.*—In the lower rows of Tables I to VI, the change per $^{\circ}\text{C}$. is expressed in per cents of the mean. These values are found by dividing the difference in facet number per $^{\circ}\text{C}$. for the

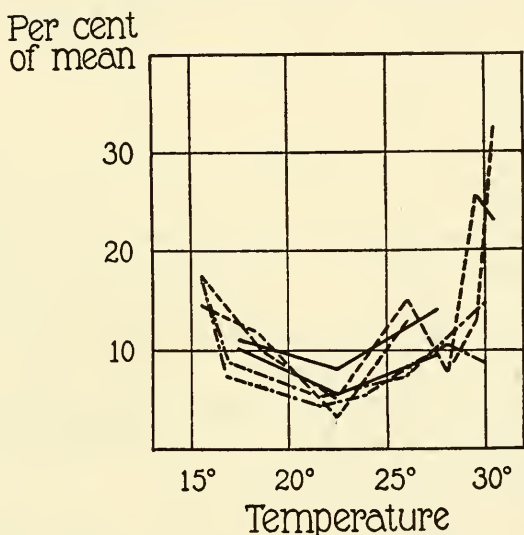


FIG. 7. Per cent of change in mean facet number accompanying a change of 1° in temperature. The solid line represents the Unselected bar stock, the broken line the Low Selected, and the dot and dash line the Ultra-bar. The upper curve in each pair represents the males.

interval by the average of the mean values for the two temperatures.

The per cent of change per $^{\circ}\text{C}$. in Ultra-bar varies from 17.39 to 4.69; in Low Selected, from 32.45 to 3.49; in Unselected, from 13.71 to 5.84. Thus the proportional change per $^{\circ}\text{C}$. is (1) not constant for all temperatures or (2) constant for the three stocks.

The average per cent change per $^{\circ}\text{C}$. in the mean of the Ultra-bar is 9.22; for Low Selected it is 14.01.

(c) *Relative Change at the Different Temperatures.*—All three stocks show one feature in common. The extreme high (29–31°) and low (15–17.5°) temperatures produce the greatest changes in facet number per °C. when the differences are expressed in per cent of the mean. The lowest proportional changes are produced by the intermediate temperature at or near 23° (Fig. 7).

Except for this phenomenon of increased change at the extreme temperatures, the difference in facet number accompanying a change of 1°C. is roughly proportional (about 10 per cent) to the mean facet number. Thus a decrease in the mean, whether produced by germinal or environmental factors, produces a corresponding decrease in the temperature increment.

Application of van't Hoff's Law to Temperature Effect on Facet Number.

Many investigators in the past 10 years have attempted to obtain an expression for the relationship between temperature and the rates of various biological reactions. The interest in the problem has been twofold. First, in an endeavor to reduce all vital processes into terms of dynamics, sorting out the physical from the chemical, the temperature coefficients have been compared to those of various physico-chemical reactions. This has been mostly through the direct application of van't Hoff's law, which for chemical reactions means a doubling or trebling of rate for every rise of 10°C.

The practical application of temperature effects has developed another group of workers. For them van't Hoff's formula was very unsatisfactory. They give a physiological interpretation and represent the rate of vital processes as a linear function of the temperature.

The formula of van't Hoff and the physiological formula as given by Krogh are as follows:

$$\begin{aligned} v_t + 1^\circ &= v_t \times Q_1; & v_t + 10^\circ &= v_t \times Q_1^{10} = v_t \times Q_{10} \\ v_t + 1^\circ &= v_t + K_1; & v_t + 10^\circ &= v_t + 10K_1 = v_t + K_{10} \end{aligned}$$

where v is the velocity of the process, t is the temperature in °C., Q_1 and Q_{10} are the van't Hoff constants, and K_1 and K_{10} are the Krogh constants.

The Q_{10} values, when calculated directly for intervals of 10° , closely approximate the theoretical demands of van't Hoff's law (Tables VIII and IX).

When the Q_{10} values are calculated from the Q_1, Q_2 , etc., an interesting fact is discovered. In Tables X and XI, I have calculated the Q_{10} values from the formula $Q_{10} = (Q_{t_1-t_2})^{\frac{10}{t_1-t_2}}$. The values of Q_{10} vary from 5.69 to 1.60. The lower values are at the median temperatures while the higher ones are at the extremes. This is not the characteristic variation in Q_{10} for chemical reactions. The values consistently decrease in the latter with increase in temperature.

TABLE VIII.

Q_{10} Calculated Directly for Ten Degree Interval. Ultra-Bar Females.

Temperature interval.	Facet No.		Q_{10}
$^\circ\text{C.}$			
15-25	51.51	25.24	2.04
17-27	40.14	21.27	1.89
20-30	32.59	15.90	2.03

TABLE IX.

Q_{10} Calculated Directly for Ten Degree Interval. Low Selected Bar Females.

Temperature interval.	Facet No.		Q_{10}
$^\circ\text{C.}$			
15-25	189.00	74.25	2.54
20-30	98.88	36.56	2.79

In Fig. 8 the curve for the facet numbers in the Ultra-bar females is superimposed on a theoretical van't Hoff curve. In the latter the rate at 10°C. is arbitrarily taken as 10; from which at 20°C. it becomes 20 and at 30°C. it is 40. Intermediate values are interpolated. The experimental curve is fitted to the other by taking the value of "facets" at 20°C. as 20 and then applying the $Q_{t_1-t_2}$ as given in Table X. It is plainly evident that the experimental curve for the effect of temperature upon facet number is something more than a chance approximation of the theoretical van't Hoff curve.

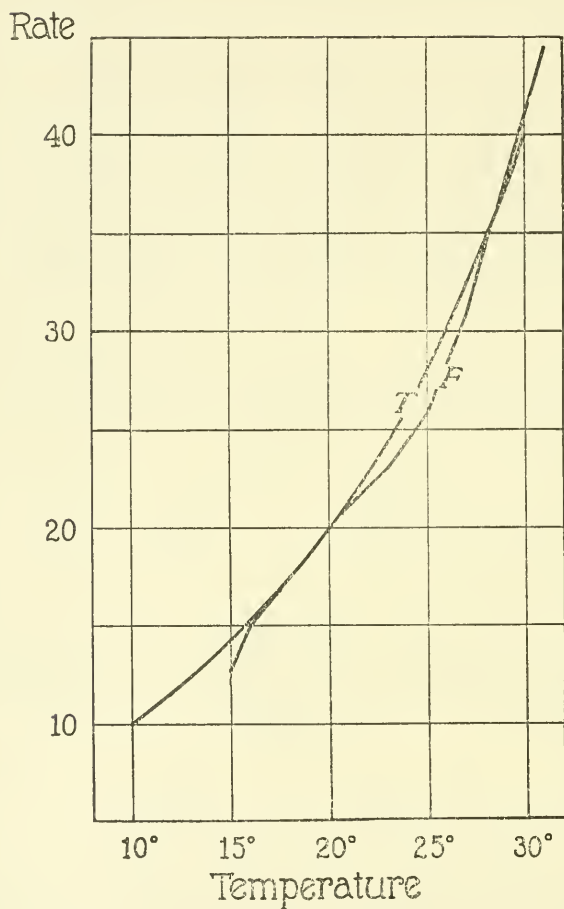


FIG. 8. A theoretical van't Hoff curve (T). The rate at 10° is arbitrarily taken as 10, at 20° it becomes 20, and at 30° it is 40. The reciprocal of the Ultra-bar female facet number curve (F) is shown superimposed. The curves are thrown into juxtaposition by taking the value 20 for facets at 20° and then calculating the remaining values by applying the $Q_{t_1-t_2}$ values, as given in Table X.

TABLE X.

Q₁₀ (Calculated from Q_{t₁-t₂) for Facet Number in Ultra-Bar Females.}

Temperature.	Mean facet No.	$Q_{t_1-t_2} = \frac{M_1}{M_2}$	Q ₁₀
°C.			
15	51.51		
16	43.27	1.19	5.69
17.5	38.57	1.12	2.15
20	32.59	1.18	1.96
23	28.30	1.15	1.60
25	25.24	1.12	1.77
27	21.27	1.18	2.36
29	17.23	1.23	2.86
31	14.57	1.18	2.33

TABLE XI.

Q₁₀ (Calculated from Q_{t₁-t₂) for Facet Number in Low Selected Females.}

Temperature.	Mean facet No.	$Q_{t_1-t_2} = \frac{M_1}{M_2}$	Q ₁₀
°C.			
15	189.00		
16	158.20	1.19	5.92
17.5	127.27	1.24	4.26
20	98.88	1.28	2.74
25	74.25	1.33	1.77
27	55.13	1.34	4.43
29	47.40	1.16	2.12
30	36.56	1.29	13.36
31	28.85	1.26	10.66

Temperature Effect on the Rate of Development.

The time-temperature curves, representing the number of days necessary to pass through a given stage in the life history, give an interesting set of data with which to compare the facet curves. Table XII gives the number of days at the respective temperatures necessary to complete the egg, larval, and pupal stages. Loeb has treated in full the subject of the length of the various metamorphic phases in *Drosophila* as affected by temperature. As my results are consistent with his in as far as the type of curve is concerned, I shall say nothing

further on this point. My values for the length of the egg-larval-pupal period do not coincide in some particulars with his, due to a difference in technique. My flies were subjected to the experimental temperatures immediately upon mating.

With my cultural methods, I was unable to get the Ultra-barstock to develop beyond pupation at 33°C. The data given in the developmental curve for this temperature are for wild full-eyed stock. There is some variation in the length of the periods as shown by the individual cultures. All sets made up at the same time and with the same food, however, give the type of curve shown by the averages (Table XII).

TABLE XII.

Effect of Temperature upon the Length of the Immature Period (Egg-Larval-Pupal) in Drosophila.

Temperature.	Mean length in days of immature period.	Reciprocal or rate per day.
°C.		
15	31.87	0.0313
16	22.93	0.0436
17.5	19.20	0.0521
20	13.62	0.0734
23	11.75	0.0851
25	10.38	0.0964
27	9.22	0.1084
29	8.27	0.1209
30	9.03	0.1107
31	9.21	0.1085
33	9.42	0.1062

In Fig. 9 is shown the number of days from mating of the parents to the emergence of the offspring from pupation. The reciprocal, or rate per day, curve is also given. This rate curve gives approximately a straight line between 15 and 29°C. Beyond 29° it turns down, thus showing an optimum.

This type of temperature curve is found for nearly all physiological reactions. Krogh has recalculated Loeb's data and has shown that they represent the time-temperature relations for the rate of segmentation of the sea urchin eggs far better than the van't Hoff formula. In the experimental data of various workers he has demonstrated this

type of curve. He has demonstrated the straight line curve in the rate of CO_2 production by *Tenebrio* larvæ. Sanderson, Headlee, Peairs, and many others have shown that all the various phases of insect metabolism investigated follow this principle. Lehenbauer, as the

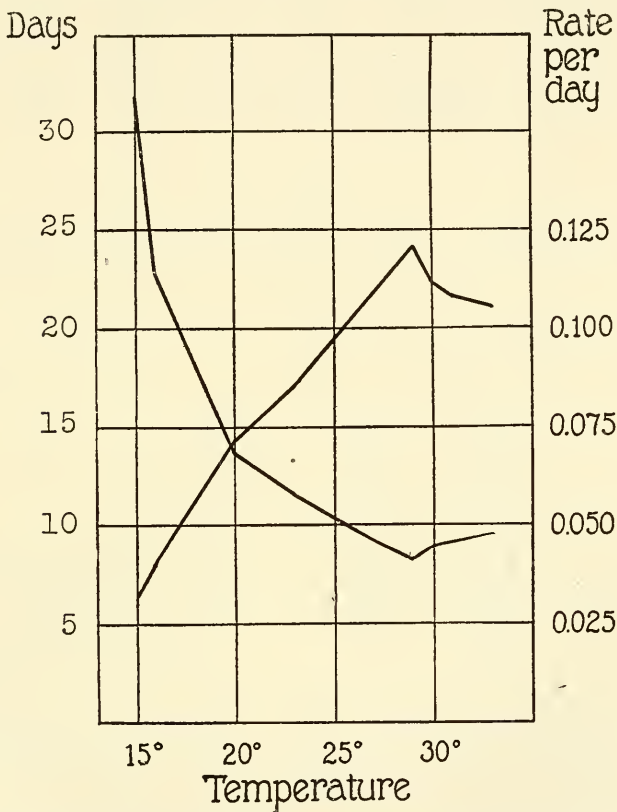


FIG. 9. Temperature effect on the length of the immature stages (egg, larval, and pupal) and on the rate of development. The curve that starts at the top to the left represents the number of days from the mating of the parents to the emergence from pupation of the offspring. The reciprocal represents the percentage of total development completed in 1 day.

most recent worker on rate of plant growth, has shown the same relations for maize seedlings. An examination of many curves dealing with the effect of temperature on the rate of growth and the degree

of infection of parasitic fungi demonstrates the same principle here. Simpson and Rasmussen's data for the rate of coagulation of blood give a similar curve.

Snyder, Fallas, and Elmendorf, dealing with the rate of heart beat of the cat, maintain that this reaction is a logarithmic and not a linear function of the temperature. Groves has applied a similar formula to the length of life of seeds at various temperatures. Loeb has worked out the temperature relations for the total length of life of *Drosophila* and has applied an exponential curve. He compromises

TABLE XIII.

Q_{10} (Calculated from $Q_{t_2-t_1}$) for the Velocity of Development of the Immaturity Stages of *Drosophila*.

Temperature.	Rate per day.	$Q_{t_2-t_1} = \frac{V_2}{V_1}$	Q_{10}	$Q_{t_1-t_2} = \frac{V_1}{V_2}$	Q_{10}
°C.					
15	0.0313	1.392	27.31		
16	0.0436	1.195	3.28		
17.5	0.0521	1.409	3.94		
20	0.0734	1.313	1.72		
25	0.0964	1.124	1.79		
27	0.1084	1.115	1.72		
29	0.1209	0.915	0.41	-1.092	-2.41
30	0.1107	0.980	0.82	-1.020	-1.22
31	0.1085	0.978	0.89	-1.021	-1.11
33	0.1062				

on the larval-pupal period, however, and admits the straight line relations there.

An important feature of the straight line temperature-rate curve is that it holds only between certain temperatures. As already pointed out for the high temperatures, the rate decreases with increase in temperature above the optimum. Another feature of the straight line curve is that at the lower temperatures the rates are higher than they theoretically should be. The first feature is noted in these experiments; the second was not, as the lowest experimental temperature was not the minimum for development. In Table XIII are shown the Q_{10} values for the rate of development as calculated from

the $Q_{t_2-t_1}$ experimental values. For the 16–15° interval, $Q_{10} = 27.31$; for the 17.5–16° interval it is 3.28; there is a decrease in the value from here as the temperature increases until at 33–31° the value is 0.89.

I wish to point out here that above 29° the values of Q_{10} really become negative.

At 29 – 30°	$Q_{10} = -2.41$
“ 30 – 31°	$Q_{10} = -1.22$
“ 31 – 33°	$Q_{10} = -1.11$

Obviously Q_{10} has no practical value for these reactions since its value may lie anywhere between 27.31 and -2.41 . A glance at Lehenbauer's data on the rate of growth of maize seedlings shows even a greater departure from the theoretical 2–3 value of Q_{10} . Here it ranges from 32 at 15–13° to -428.2 at 42–45°C. Exceedingly high temperature coefficients are suggestive of enzymatic reactions and toxicity effects while the negative values suggest physical processes.

Relation of the Temperature Effects on the Length of the Developmental Periods to Those on Facet Number.

We have seen that the facet curve can be superimposed on the theoretical Q_{10} curve with a closeness that can hardly be attributed to chance. The next obvious thing is to see how closely the facet and the metamorphic curves agree.

In Fig. 10, the facet curves for the Low Selected bar stocks are superimposed on the development curve. From 15–25° the two sets of curves approximate each other very closely. Above 25°, the metamorphic curve decreases less rapidly, until above 29° it begins to turn up again. The facet curves continue to decrease at their initial rate.

The reciprocal curves are of particular interest. That for the rate of development gives approximately a straight line between 15 and 29°. It likewise shows an optimum at 29° with a subsequent decrease in rate and further increase in temperature. The reciprocal of the facet curve gives an exponential curve, without a decrease in rate at the upper temperatures. The lower part of this curve approximates the straight line features of the metamorphic curve. In other words,

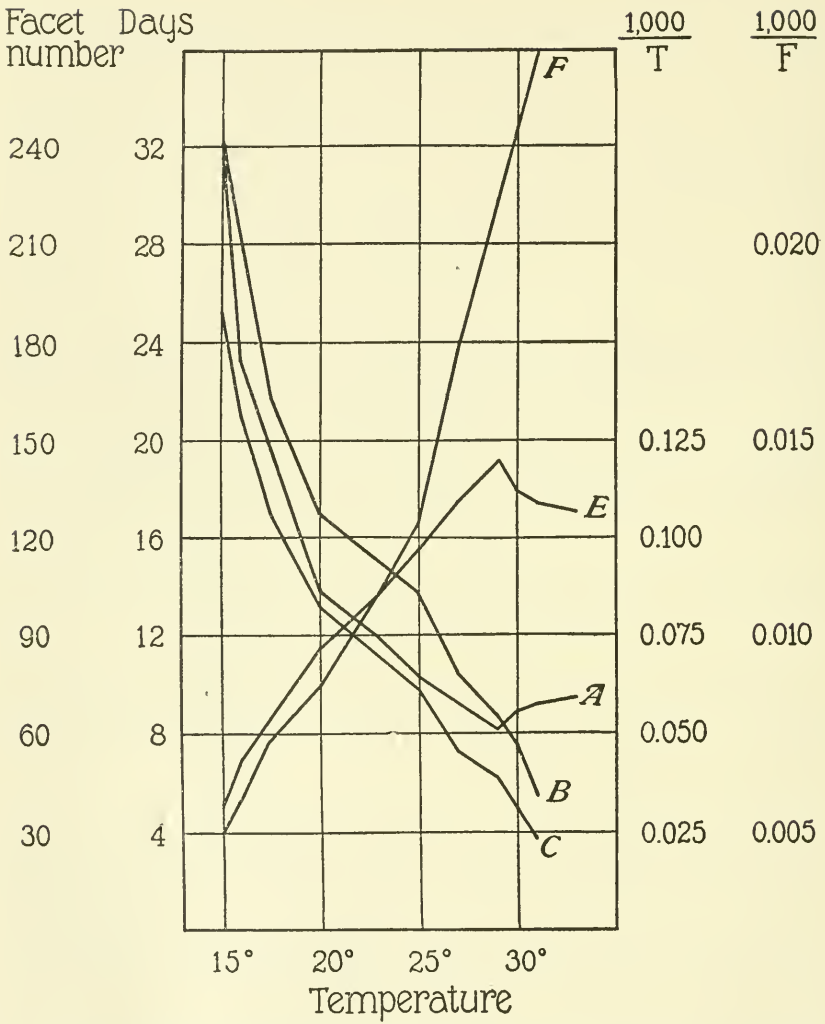


FIG. 10. The Low Selected bar stock facet curves superimposed on the curve for the length of development of the immature period. These curves are thrown into juxtaposition by arranging the values so that the developmental curve A lies about half way between the male facet curve B and the female facet curve C. The reciprocal curve E for the rate of development and the reciprocal female facet number curve F are likewise brought into comparison by arranging the values for each.

the length of the developmental period and the number of facets are correlated up to the point where the time-temperature relations for metamorphosis begin to fail.

This leads to the question; Is the number of facets dependent upon the length of the immature stage?

TABLE XIV.

Experiment 6. Unselected Bar Stock. Larvæ Subjected to 30° for First Part of Development; Remainder at 15°.

No. of days at 30° before removal to 15°.	1	2	3	4	5	6	7							
Facet No. classes.	Distribution of individual counts.													
15-44					4	1	7	3	4	2	10	3		
45-74					4	7		10			2	7		
75-104					6	2	4	2	1					
105-134					5	1	3	2						
135-164				2	1	1	2	1						
165-194				3	4	5	1	3	5					
195-224	3	1	7	1	10	4	6	3						
225-254	6	3		2	11	6		2						
255-284	1	1	1	3	2	6	2	1						
285-314		4			1	4		4						
315-344		1				2								
Mean facet No.	230	279	216	251	221	246	152	224	93	95	44	59	36	48
Total No. of days to complete immature stages.	25		24		23		22		22		20		18	
Control stocks.														
At 15° throughout.									At 30° throughout.					
Facet No.						213	269	39			73			
Total No. of days in period						31.87			9.02					

In the previous experiments it is obvious that there is a certain degree of correlation between the facet number and the length of the egg-larval-pupal period. Are these two phenomena directly dependent or are they separately affected by a third common factor? Experiment 6 was designed for another purpose but it supplies data here.

In Table XIV is given the distribution of facet counts in Experiment 6 in which the successive bottles, made up by changing the same par-

ents daily, were treated to varying periods at 30° before subsequent development at 15°. The upper row in the table gives the number of days at 30°. Below the class distributions are given the mean facet values for males and females. In the lower row is given the number of days required to complete the immature stage.

It is evident that there is no direct causal relation between the number of days taken to complete the immature stage and the number of facets. 25, 24, and 23 days give practically the same facet counts that are obtained when total development is passed at 15°. In the last case 31 days are required to complete the immature stages. Again, those flies hatching at 18 and 20 days are in the same facet classes as those hatching in the stock experiments in 9 days at 30°.

Why, then, is there an apparent correlation between the facet count and the length of the immature period when the latter is passed throughout at one temperature? We shall return to this question at the end of the succeeding part of this study.³

The author wishes to express gratitude to Dr. Charles Zeleny, under whose direction the work was done.

Acknowledgments are also due to Doctor V. E. Shelford for many valuable suggestions and to Doctor G. Dietrichson for the loan of apparatus.

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³ Krafka, J., Jr., *J. Gen. Physiol.*, 1919-20, ii, in press.

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THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

VOLUME II, NO. 5

MAY 20, 1920



PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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CORRECTION.

On page 243, Vol. ii, No. 3, January 20, 1920, line 12 (counting each equation as a line) for *a photochemical reaction* read *a reversible photochemical reaction.*

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THE EFFECT OF TEMPERATURE UPON FACET
NUMBER IN THE BAR-EYED MUTANT OF
DROSOPHILA.*

PART II.

BY JOSEPH KRAFKA, JR.

(From the Zoological Laboratory of the University of Illinois, Urbana.)

(Received for publication, December 30, 1919.)

*Determination of the Period during Which Temperature is Effective in
Modifying the Facet Number.*

Preliminary Experiments on Unselected Bar Stock at 30 and 15°.

A preliminary experiment was planned to determine whether temperature had an effect throughout the immature stages or whether it was limited to a specific phase of development. In Table XIV¹ are shown the results of subjecting successive cultures for the 1st day, first 2 days, first 3 days, and so on to 30° before subsequent development at 15°. 3, 2, and 1 days at 30° give the same facet number as those raised at 15° throughout. The early days of larval life may be spent at high temperatures without effect on the facet number.

6 and 7 days at 30° followed by transfer to 15° show that the number of facets had been determined prior to the transfer, as indicated by the fact that all the counts come well within the range of the stock counts at 30°.

Next, if we consider only those counts made on the 1st day of hatching, the bottles that were kept at 30° for the first 4 and 5 days show only the facet counts characteristic of the 30° stocks. The counts made on the flies hatching on the 2nd and 3rd days are inter-

* Contribution from the Zoological Laboratory of the University of Illinois, No. 148.

¹ Krafka, J., Jr., *J. Gen. Physiol.*, 1919-20, ii, 409.

mediate between the 30 and 15° stocks. These intermediates were to be expected. The parents were not removed from the bottles until the end of the first 24 hours. Hence some of the larvæ may have been 24 hours older than others. Those 4 days old had already passed the point X in development during which the facet number-determining reaction is going on. They are also the first individuals in the bottle to hatch. Those hatching later were not so far along in development and hence were affected by the transfer to 15°.

Obviously temperature is not capable of modifying the facet number throughout the immature stages, but is limited to a definite stage in development.

Subsequent experiments on Ultra-bar consider these points more in detail.

Effect of Temperature during the Pupal Period.

Experiments of the same type as the preceding were carried out on Ultra-bar at 27 and 15°. We will consider the effects of temperature during the pupal period first. Subjecting the cultures to 27° for 5, 6, 7, 8, and 9 days before subsequent removal to 15° gave counts which are characteristic of the 27° stocks both in mean and in individual variation. The first four cultures had pupated before the transfer to the colder temperature was made. In the last one the imago had begun to emerge from pupation as the transfer from 27 to 15° was made. Obviously in the last case a change of temperature would have no effect, since there is no further change in the number of facets after the adult is once formed.

From the distribution of the counts in Table XV it is just as clear that subjecting the immature stage to low temperature after pupation has no effect on the facet number. This is a particularly important point, since the facets themselves first become evident only toward the end of the pupal period. The reaction which has determined the number of facets that are to form has greatly preceded the actual formation of these facets (Fig. 11).

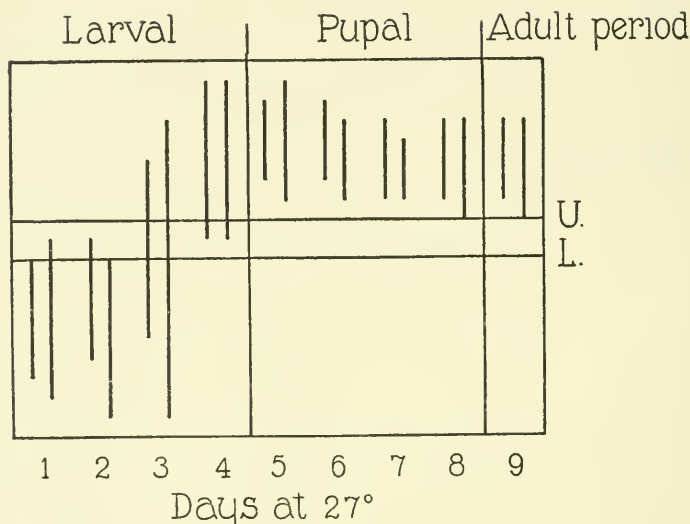


FIG. 11. Effect on the distribution of facet counts as a result of subjecting successive cultures from the same parentage of Ultra-bar to varying periods of time at 27° before subsequent development at 15°. After mating the first bottle was put at 27° for 24 hours. The parents were then removed to the second bottle. The first bottle was allowed to complete development at 15°. At the end of 24 hours, the parents were removed to a third bottle, etc. The second bottle remained at 27° for 48 hours, and completed development at 15°. The third bottle remained at 27° for the first 3 days, etc. The first vertical line of a pair represents the range of the female counts, the second is that of the males. Those cultures to the left of the first full vertical line were in the larval stage when the transfer from 27 to 15° was made. Those between the two full vertical lines were in the pupal stage when the transfer was made (see Table XV). U. is the upper range of 27° stock counts; L., the lower range of 15° stock counts.

Effect of Temperature during the Larval Period. Initiation and Duration of the Effective Period at 27°.

An examination of Table XV shows that a temperature of 27°C. for 1 or 2 days at the beginning of the larval period has no effect on the facet number, as only three individuals out of 123 are slightly outside the range of the stock experiments at 15°.

Of those that passed the first 3 days at 27°, some had developed beyond the point X, at which facet number is determined, since their counts are characteristic of the 27° stock. Some were just in the effective period when the transfer from 27 to 15° was made, as shown by the fact that the counts are intermediate between those characteristic of the 27 and those of the 15° stocks. Some individuals were not quite so far along and hence the whole facet number-determining reaction was carried out at 15°.

4 days at 27° brought nearly all individuals through the effective period. Four individuals out of 104 are slightly above the upper range of the 27° stock counts. We may conclude that the stage in development, at which the facet number is being determined, is passed prior to the end of 4 days at 27°. Likewise this stage is not reached by the end of 2 days at 27°.

To define this period more closely Experiment 59 was planned. After many unsuccessful attempts to get a series in which the eggs were not more than 1 hour apart in age, the present series was carried through. Here the parents were allowed to lay eggs during a period of 12 hours. This series includes the following number of days at 27° with subsequent removal to 15°; 1 day (24 hours), 2, $2\frac{3}{4}$, 3, $3\frac{1}{4}$, $3\frac{1}{2}$, $3\frac{3}{4}$, and 4 days. The results are given in Table XVI and in Fig. 12.

This experiment bears out the previous one in that 1, 2, $2\frac{3}{4}$, and 3 days at 27° did not bring the larvæ up to the point X. This phase of development is initiated between 3 and $3\frac{1}{4}$ days as shown by the intermediate condition of the counts of the latter. These are predominated by the lower temperature. $3\frac{1}{2}$ days at 27° give a preponderance of individuals with the 27° count. $3\frac{3}{4}$ days at 27° have brought all but one individual through the effective period. After 4 days at 27°, all individuals had completed this stage in de-

velopment and further changes in temperature had no effect on the facet number thereafter.

A second culture, allowed to develop 3 days at 27°, demonstrates individual variation in rate among separate bottles. In this culture the effective period was passed by thirteen individuals at 27°,

TABLE XVI.

Experiment 59, Showing Distribution of Facet Numbers. Larvæ Were Allowed to Complete Part Development at 27° and Were then Transferred to 15° for Remainder.

No. of days at 27° before transfer to 15°.	1	2	2½	3 (I)	3 (II)	3½	3¾	3½	4	27° Control.
Facet class.	Distribution of individuals in separate bottles.									
15-18							5	2	3	3
19-22					2		3	1	8	23
23-26					3		7	3	11	38
27-30					4	1	4	10	3	32
31-34	Upper range of 27° stock.				2	2	4	1	8	3
35-38					4	7	3	1	12	
39-42			1	1	9	5	3	2	12	
									9	Lower range of 15° stock.
43-46	1	1	2	1	1	7	3	7	3	5
47-50	4	6	2	4	1	9	4	4	2	6
51-54	17	2	11	3	9	11	9	3	13	7
55-58	12	7	4	4	8	9	5	1	1	3
59-62	6	7	6	6	6	5	2	4	2	4
63-66	4	7	2	3	3	6	2	11	2	3
67-70	3	10	2	5	2	1	7	3	1	
71-74	3	5	1			4	2		1	
75-78	2									
79-82					1					

before the transfer was made; and in many others the counts are intermediate showing that the reaction was going on at the time of the transfer from 27 to 15°.

A control mating by the same parentage as the foregoing series was reared at 27°. It shows the normal distribution of the 27° stock.

According to this and the previous experiment, the reaction, which determines the number of facets and whose rate is subject to temperature modification, is initiated at 27° at or near the end of 3 days of development. Furthermore, it is practically over at the end of $3\frac{3}{4}$ days, making the effective period of less than 18 hours duration.

The actual time during which the reaction which determines the number of facets takes place is doubtless much shorter than this for

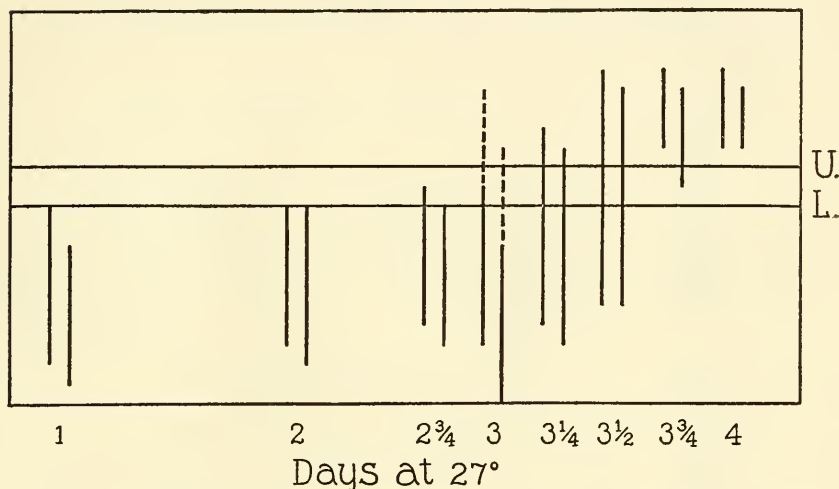


FIG. 12. Effect on the distribution and range in the Ultra-bar as a result of subjecting successive bottles to 27° for 1, 2, $2\frac{3}{4}$, 3, $3\frac{1}{4}$, $3\frac{1}{2}$, $3\frac{3}{4}$, and 4 days, respectively, before removal to 15° (see Table XVI). Broken line represents extension of range in a second culture kept at 27° for 3 days. U. is the upper range of 27° stock counts; L., the lower range of 15° stock counts.

the individual, since under the conditions of the experiment we are dealing with material that may differ 12 hours in age.

Initiation and Duration of the Effective Period at 15° .

Experiment 62, of the same type as the preceding except that the conditions were reversed, was carried out to determine when the effective period was initiated at 15° . The same parents were used as in Experiment 59. The cultures were made up at 27° and left

for 24 hours. The parents were then removed to the next set of bottles, while the bottles containing the eggs and larvæ were transferred to 15°. Here they were left for the number of days indicated in Table XVII and then returned to 27° to complete development.

TABLE XVII.

Experiment 62, Showing Distribution of Facet Numbers. Larvæ Were Allowed to Complete Part of Development at 15° and Were then Transferred to 27° for Remainder.

No. of days at 15° before transfer to 27°*.....	1	2	3	4	5	6	7	8
Facet class.	Distribution of individuals in separate bottles.							
11-14		4			2	1	1	
15-18	5	1	8		5	1	7	3
19-22	15	16	35	16	25	25	23	15
23-26	23	26	50	63	15	18	26	32
27-30	2	12	7	21	3	9	4	12
31-34	Upper range of 27° stock counts.				2	1		1
35-38								
39-42	Lower range of 15° stock counts.							1
43-46								1
47-50								1
51-54								1
55-58								
59-62								1
63-66								
67-70								1
71-74								
75-78								
79-82								

* The bottles were at 27° the first 24 hrs.; then removed to 15° for the number of days indicated before return to 27°. Parents are the same as in Experiment 59.

It is plainly evident that the effective period is not initiated at the end of 3 days as at 27°. The point X is not reached even at the end of 7 days at 15° plus the 1st day at 27°. In Experiment 62, the change comes on the 8th day at 15° (Fig. 13). Unfortunately this experiment was not planned to cover a longer period; a second experiment was therefore started.

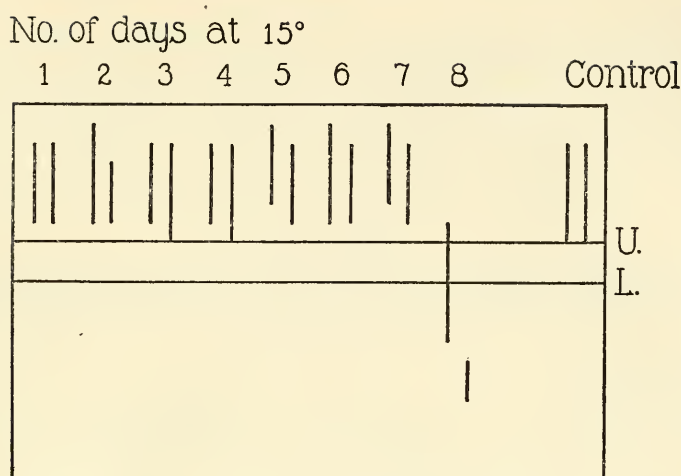


FIG. 13. Effect on the range of facet counts in Ultra-bar as a result of subjecting successive cultures from the same parents for varying periods to 15° before subsequent development at 27° (see Table XVII). U. is the upper range of 27° stock counts; L., the lower range of 15° stock counts.

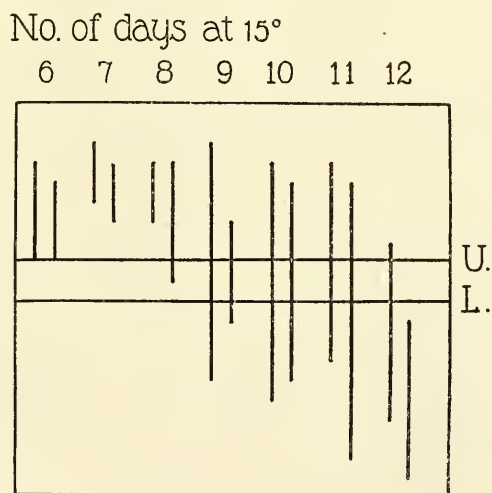


FIG. 14. Duration of the effective period at 15°. Effect on the range of facet counts as a result of subjecting successive cultures from the same parentage for varying periods of time to 15° before subsequent development at 27° (see Table XVIII). U. is the upper range of 27° stock counts; L., the lower range of 15° stock counts.

Experiment 72 supplies the data on the length of the period at 15° (Table XVIII and Fig. 14). As pointed out for Experiment 59 there may be a slight difference in the rates of the individual bottles. Experiments 62 and 72 show such a variation. In Experiment 62, the period was initiated on the 8th day. In Experiment 72, only two

TABLE XVIII.

Experiment 72, Showing the Distribution of Facet Numbers. Larvæ Were Allowed to Complete Part Development at 15° and Were then Transferred to 27° for the Remainder.

No. of days at 15° before return to 27°.*	6	7	8	9	10	11	12	
Facet class.	Distribution of individuals in separate bottles.							
11-14			1		2			
15-18	1		2	3	2	1	1	
19-22	6	6	4	4	5	3	0	3
23-26	1	2		2	5	1	2	3
27-30					2	4	4	2
31-34	1	1			2	3	2	1
								Upper range of 27° stock.
35-38					1	3	1	1
39-42					1	2	2	1
								Lower range of 15° stock.
43-46					1	1	1	2
47-50							2	1
51-54						2	1	2
55-58					1		2	1
59-62						1		6
63-66							2	3
67-70								14
71-74							1	6
75-78								1

* All these cultures were at 27° for the first 24 hrs. They were then removed to 15° for the number of days indicated at the top of the table and allowed to complete development at 27°.

individuals had passed the effective period at 15° when the transfer was made from 15 to 27°. After 9, 10, and 11 days an increasing number of individuals show the 15° count, and after 12 days only five of 94 individuals had not completed the facet number-determining reaction when the transfer was made.

As there may be 24 hours difference in age among the individuals in a bottle, the period at 15° is practically limited to the 9th, 10th, and 11th days, or the length of the period at 15° is about 72 hours.

Comparison of the Length of the Period during Which Temperature is Effective on Facet Number with the Total Length of the Immature Stage.

It has been shown that the period during which temperature is effective is initiated at two very remote time intervals when development is carried out at 15 and 27°, respectively. Does this period represent a definite stage in development?

The total number of days required to complete development of the immature stages at 15° is 31.87, while at 27° it is 9.21. This gives a daily rate of 3.13 per cent total development at 15° and 10.86 per cent total development at 27°. With these rates we may calculate the point X.

3 days at 27°	$3 \times 10.86 = 32.58$	per cent
8 " " 15°	$8 \times 3.13 = 25.04$	" "
1 day " 27°	$1 \times 10.86 = 10.86$	" "
	35.90	" "

In other words, the reaction which determines the number of facets that will develop in the adult is initiated at the completion of 32 to 36 per cent of immature development.

Comparing now the length of the periods during which this reaction is going on at 15 and at 27° with the total length of the immature stages at those temperatures, we find 18 hours at 27° and 72 hours at 15° as opposed to 9.21 days at 27° and 31.87 days at 15°. These ratios are a fair approximation considering the experimental conditions.

Expressing the length of the effective period in percentage of the total development and adding the results to those for the initiation of the period, we find that the reaction which determines the number of facets starts at the completion of 32 per cent of development and ends with the completion of 45 per cent.

Thus the length of a particular phase of development is proportional to that of any other phase. Reciprocally, the rate of a given

reaction such as that which determines facet number is proportional to the rate of general metabolism.

In the first part of this study we noted a correlation between facet number and length of the immature stage when the latter was completed at a single temperature. Subsequently it appeared that facet number and the total length of the immature period were independent. Why do we find this apparent correlation?

The facet-determining reaction has been shown to be of relatively short duration. It is obvious that a change in temperature following its completion could affect the total length of the immature period without affecting the facet number.

We may conclude that the number of facets and the length of the immature period are not directly dependent but rather that the former is determined by a specific reaction the rate of which is correlated with that of the general metabolic processes extant while this particular phase of development is going on.

THE EFFECT OF TEMPERATURE UPON FACET NUMBER IN THE BAR-EYED MUTANT OF DROSOPHILA.*

PART III.

By JOSEPH KRAFKA, JR.

(From the Zoological Laboratory of the University of Illinois, Urbana.)

(Received for publication, December 30, 1919.)

The Effect of Temperature upon Variability.

Coefficient of Variability at Different Temperatures.

This study gives some interesting data on the question of individual variability at the various temperatures. The distribution of the counts of Ultra-bar are given in Table XIX. Here the class size is 1 facet. Particular attention is called to the normal distribution in all cases.

An argument for the genetic stability of the Ultra-bar stock is found in the relatively infrequent occurrence of individuals outside the bounds of normal distribution. At 27° a single 55 facet male and at 16° a 72 facet male are the only two extremely wide departures.

The mean, standard deviation, and coefficient of variability for all three stocks are given in Tables XX to XXII. Two things are apparent: (1) variability increases with the temperature when measured by the coefficient of variability; and (2) the variability of the Ultra-bar stock is much lower than that of the other two stocks. Both these generalizations have their exceptions. Their reliability as compared to statements made by other writers will be discussed later.

* Contribution from the Zoological Laboratory of the University of Illinois, No. 148.

TABLE XIX.

Showing the Distribution of the Facet Counts in the Stock Experiments on Ultra-Bar.

Facet No.	Temperature.																	
	15	16	17.5	20	23	25	27	29	31	15	16	17.5	20	23	25	27	29	31
	Females.									Males.								
5								1	1									1
6									1									1
7									1								1	4
8									2									4
9								2	3									6
10								5	4								6	8
11								8	6								1	15
12							2	14	14								9	20
13							5	19	16							1	10	16
14						1	2	46	21							1	17	26
15						2	11	74	19							1	36	22
16						2	11	63	15							1	40	22
17						1	22	77	11							8	55	16
18						5	38	76	10							20	84	7
19						5	58	72	8						1	26	96	9
20						14	59	52	2						6	34	83	3
21				1	1	26	66	48	2						7	53	82	3
22				2	1	44	61	29	0						14	67	55	2
23				1	5	32	38	17	1				2	23	59	25		1
24				3	8	61	38	12	1				1	27	55	20		1
25				2	9	59	28	10					3	54	55	9		
26				4	8	72	17	3				1	3	51	57	10		
27				2	7	58	15	2				0	3	58	30	2		
28				7	11	50	9					2	5	59	22			
29				6	10	38	8					4	11	59	21			
30				7	10	13						5	8	39	9			
31		1	3	12	7	8	1					6	5	45	8			
32		0	5	13	6	7						7	12	25	6			
33		2	10	8	3	2	1					7	15	12	2			
34		0	8	10	2							9	12	9	1			
35		3	9	6	3							9	5	5	0			
36		4	8	4	2						1	4	3	1				
37		3	12	6	1					2	2	12	3	1				
38		6	10	3			1			1	1	7	2					
39	1	10	11	5						0	2	8	1					
40	0	3	3	1						1	5	4						
41	1	8	7	4						3	9	14						
42	2	11	0	1						1	4	10	8					
43	4	7	5	0						0	3	10	6					
44	3	10	6	3						1	2	7	4					
45	3	5	3	0						1	1	10	0					

TABLE XIX—*Concluded.*

Facet No.	Temperature.																	
	15	16	17.5	20	23	25	27	29	31	15	16	17.5	20	23	25	27	29	31
	Females.									Males.								
46	3	3	0	0						0	0	8	1					
47	3	4	2	1						0	6	9	0					
48	3	4	4							0	10	11						
49	5	2	3							2	7	2						
50	1	2	1							1	5	1						
51	1	3	0							2	9	1						
52	2	2	1							3	9	1	1					
53	2	2	1							3	6	4						
54	5	2								2	4	3	1					
55	2	0								2	7	1				1		
56	1	1								0	4	0						
57	1	1								5	5	1						
58	3	1								4	5	0						
59	0	0								4	2	1						
60	1	0								2	1							
61	1	1								3	3							
62	2									4	1							
63	0									4	1							
64	0									2								
65	1									0								
66	1									1								
67	1									4								
68	0									3								
69	1									1								
70	0									0								
71	0									3								
72	1									1	1							
73	0									0								
74										1								
75										1								
76										1								
77										1								
78																		
79																		
80																		
81																		
82																		
83										1								
84																		
85										1								

TABLE XX.

Coefficient of Variability at the Various Temperatures. Unselected Bar Stock. Females.

Temperature.	Mean facet No.	Standard deviation.	Coefficient of variability.
°C.			
15	213.67 \pm 2.12	29.78 \pm 1.48	13.94
20	122.20 \pm 1.46	23.45 \pm 1.02	19.19
25	81.08 \pm 1.21	19.19 \pm 0.85	23.67
30	39.66 \pm 0.87	12.73 \pm 0.61	32.10

TABLE XXI.

Coefficient of Variability at Various Temperatures. Low Selected Bar Stock. Females.

Temperature.	Mean facet No.	Standard deviation.	Coefficient of variability.
°C.			
15	189.00 \pm 2.26	29.77 \pm 1.58	15.75
16	158.20 \pm 1.56	23.58 \pm 1.10	14.90
17.5	127.27 \pm 1.49	22.19 \pm 1.04	17.43
20	98.88 \pm 1.27	19.23 \pm 0.90	19.45
25	74.25 \pm 0.87	16.55 \pm 0.61	22.29
27	55.13 \pm 0.73	13.88 \pm 0.52	25.18
29	47.40 \pm 0.63	9.31 \pm 0.44	19.64
30	36.56 \pm 0.79	9.37 \pm 0.56	25.63
31	28.85 \pm 0.41	6.07 \pm 0.29	21.04

TABLE XXII.

Coefficient of Variability at Various Temperatures. Ultra-Bar Stock. Females.

Temperature.	Mean facet No.	Standard deviation.	Coefficient of variability.
°C.			
15	51.51 \pm 0.70	7.69 \pm 0.49	14.93
16	43.27 \pm 0.39	5.83 \pm 0.28	13.47
17.5	38.57 \pm 0.32	5.03 \pm 0.23	13.04
20	32.59 \pm 0.32	5.06 \pm 0.22	15.53
23	28.30 \pm 0.24	3.52 \pm 0.17	12.44
25	25.24 \pm 0.09	3.12 \pm 0.07	12.36
27	21.27 \pm 0.10	3.35 \pm 0.07	15.75
29	17.23 \pm 0.08	3.23 \pm 0.06	18.74
31	14.57 \pm 0.18	3.16 \pm 0.13	21.69

The Sex Coefficient at Different Temperatures.

A marked sexual difference exists in all these experiments. The average value for the ratio between the mean facet number of the females and that of the males is 0.791. Temperature has no consistent effect in altering this ratio.

An explanation of the sexual difference is to be sought in the fact that we are dealing with a sex-linked factor. On the chromosome hypothesis, a double dose of the restricting factor is present in the female while only a single dose is present in the male.

*Inheritance of Temperature Effects.**Comparison of Offspring Raised at 27°, from Parents Reared at 15, 20, and 27°, Respectively.*

The interest in this phase of the work is both practical and theoretical. To preclude any inherited effect in the stock experiments, care was taken to keep the parent stocks at 27°. To determine whether or not there was an inherited effect, Experiment 51 was designed. Flies reared for one generation in the stock experiments at 15, 20, and 27° were used as parents in cultures that were made up under conditions as nearly alike as possible. The offspring from the 15° parents, from the 20° parents, and from the 27° parents were thus allowed to develop simultaneously at 27°.

The distribution of the facet counts of the parents and the offspring is given in Table XXIII. The mean facet values and the standard deviations are given in Table XXIV. The mean facet number, the standard deviation, and the distribution of the F_1 stock reared at 27° are characteristic of the 27° stock counts although those of the parents are markedly different. There is no inherited effect of temperature upon facet number in this case.

TABLE XXIII.

Showing the Inherited Effect of Temperature. Distribution of Facet Number of Parents Reared at 15, 20, and 27°, Respectively, as Compared to Their Offspring All of Which Were Reared Simultaneously at 27°.

Facet No. classes.	Parents reared at.						Offspring reared at 27° from parents at.					
	15°		20°		27°		15°		20°		27°	
	Distribution of individuals.											
12-15					1				1		1	
16-18					7	2	1		4	2	3	2
19-21					13	6	4	3	26	11	15	7
22-24					10	11	11	15	12	28	10	14
25-27					4	9	10	14	5	15	1	8
28-30			4				2	5		1		
31-33			9					1				
34-36			6	6								
37-39	1		8	8								
40-42	1	1	2	12								
43-45	2		2	9								
46-48	2	3	1									
49-51	2											
52-54	7			2								
55-57	1	4										
58-60	1	1										
61-63	1	2										
64-66		3										
67-69		1										
70-72		1										
73-75		1										

TABLE XXIV.

Mean Facet Number and Standard Deviation of Parents Reared at 15, 20, and 27°, and of Their Offspring Reared at 27°.

Parents reared at.	Mean facet No.	Standard deviation.	Offspring reared at 27° from parents reared at.	Mean facet No.	Standard deviation.
°C.			°C.		
15	50.50 ± 0.96	6.05 ± 0.67	15	23.67 ± 0.35	2.76 ± 0.25
	58.94 ± 1.35	8.28 ± 0.95		24.85 ± 0.28	2.53 ± 0.20
20	35.50 ± 0.54	4.53 ± 0.38	20	20.85 ± 0.24	2.61 ± 0.18
	40.60 ± 0.48	4.29 ± 0.34		23.12 ± 0.24	2.80 ± 0.17
27	20.80 ± 0.34	3.02 ± 0.24	27	20.50 ± 0.29	2.39 ± 0.20
	22.96 ± 0.12	2.43 ± 0.08		22.68 ± 0.31	2.56 ± 0.22

DISCUSSION.

*Temperature as a Factor in the Mechanism of Development.**Direct Effect of Temperature upon Growth, Size, Number of Parts, Structure, and Color.*

Ordinarily temperature is not a factor capable of modifying structure to any marked extent. Certainly structural variations are in no way comparable with the variations in rate at which they are brought about. The capacity to develop specific color, size, and form is an heritable characteristic; *e.g.*, the present study involves the white-eyed mutant of *Drosophila*, of which all individuals are white-eyed regardless of the temperature at which they develop.

Many organisms, however, exhibit variations in their structural characteristics which may be considered as a direct response to temperature.

Among the earlier investigators of temperature effects on structure were Merrifield, Weismann, Standfuss, Fischer, and Dorfmeister.¹ The chief object of their experiments was the production by environmental manipulation of the various racial and polymorphic forms in the Lepidoptera.

Vernon (1895) found that the size relations between various parts of the echinoderm larvæ could be modified in response to different temperatures at which they developed. Standfuss (1895) found a reduction in the size of the imagos, as a result of rearing lepidopterous larvæ at high temperatures. This he ascribed to the indirect effect of insufficient nourishment.

Tower found that by subjecting larvæ of *Leptinotarsa decemlineata* to various temperatures he could affect the amount of pigmentation in the adult. His results are unique in that an increase from the mean temperature range of the species (22.5°C.) had the same result as a decrease. He obtained an increase in melanism down to 16° and up to 28° followed by a decrease to albinism beyond these temperatures.

Shelford found a tendency toward melanism with an increase in temperature due to the reduction in size of the unpigmented areas on the elytra of the tiger beetles.

All these reactions are complex and the materials do not lend themselves to close quantitative study. It is obvious that no simple temperature relations can be worked out for them.

¹ For a complete review, see Bachmetjew.

Examples in Which a Specific Structure Depends upon a Definite Environmental Stimulus.

In three well marked cases, specific temperatures determine the character of the organism.

Baur cites a case of *Primula sinensis* which at ordinary temperatures produces red flowers. If a plant is subjected to a temperature of 30–35°C. a few weeks before blooming, the flowers will be white. If the plant is returned to 15–20°C., the buds opening immediately will still be white but those developing later will be red. As Baur points out, white cannot be said to be inherited, red cannot be said to be inherited, but rather the capacity to produce red flowers at 15° and white ones at 30° is the thing inherited.

Hoge found a race of *Drosophila* in which one or more legs showed reduplication. Under ordinary cultural conditions only about 10 per cent of the individuals from a pure reduplicated mating showed the condition. It was later discovered that by subjecting the eggs to 9–10°C. the percentage of offspring showing the character could be raised to practically 100.

Roberts found that a temperature difference of 4–5° C. had a direct effect on the length of the wing in the mutant "vestigial" of *Drosophila*, much greater than twenty-nine generations of selection had produced.

Bar eye is a sex-linked factor that reduces the number of facets in the adult *Drosophila* from 1,000 to 70. Its Mendelian behavior is clear cut and regular. As shown in this paper, however, the number of facets produced, when this factor is present, is dependent upon the specific temperature at which a definite stage in larval development is passed.

In the first three examples only two temperatures are involved. Obviously no quantitative measure can be applied although the results are very definite.

In bar eye, observations have been made at close intervals over a range of temperature from 15–31°C. The temperature relations have been shown to approximate closely those of many physiological reactions.

In certain cases other environmental factors may be said to determine form. Morgan has shown that a definite amount of moisture is necessary for the development of the abnormal abdomen in *Drosophila*. Metz has several mutants that depend on specific cultural conditions for their recognition.

As shown by the constant results under constant external conditions, the hereditary mechanism remains the same. The reactions involved in differentiating the somatic tissues have been shown to be modifiable through various external factors, chief of which is temperature.

Consideration of the Means by Which Temperature Can Produce an Effect on Facet Number.

We have seen in the foregoing pages that facet number in the full-eyed wild fly is affected very little by temperature, while in the bar-eyed mutant there is a very marked effect. It is also evident that the effect is produced only through a relatively short period in larval life. What hypothesis can explain these facts?

Assuming a normal mechanism for facet production (A, B, C, D, E), we may say that temperature affects the rate of all the various processes involved in nearly equal amounts. The rates are increased at the higher temperatures, but at the same time the length of time of the reactions is proportionally shortened, thereby producing a constant number of facets (N) in the full eye.

In the bar eye a new condition has come about. The facet number is reduced to about $\frac{1}{4}$ that of the full eye at 15° and to about $\frac{1}{24}$ at 27° in the Low Selected line. In Ultra-bar the reduction is even greater; $\frac{1}{14}$ at 15° and $\frac{1}{40}$ at 27° .

Hypothesis 1. Reduction in Facet Number $\left(\frac{N}{a}\right)$ Due to a Reduction in the Facet-Forming Substances $\left(\frac{n}{a}\right)$.—As in the case of the full eye, increased rates at the higher temperatures with a decrease in the time of the reaction would produce a constant number of facets from a given amount of material at all temperatures.

Hypothesis 2. Surface Tension.—The number of facets in the bar-eyed stocks varies inversely with the temperature. The reaction thus has a negative temperature coefficient suggesting physical phenomena and particularly surface tension. The values of Q_{10} are far too high even to approximate the coefficients of any of the possible physical phenomena. Furthermore, if surface tension were the factor involved, we should expect it active in the full eye also, there producing similar temperature effects as in the bar.

Hypothesis 3. Reduction in the Amount of Facet-Producing Material Plus a Rate of Facet Production Independent of the Temperature.—In this case, the independent rate working through an increased time interval at the low temperatures would produce the greater number of facets as observed in the bar eye. However, when we apply the independent rate to full eye we should obtain a proportional difference at high and low temperatures, a condition shown not to exist.

Hypothesis 4. Considering the Reduction in Facet Number to be Due to an Inhibitor.—Assuming the inhibitor to be constant in amount for all temperatures, then if it follows the time-temperature laws of the other metabolic reactions, its rate will be decreased at the low temperatures, while the time during which it acts will be proportionally lengthened. At the higher temperatures the rate will increase while the time is proportionally shortened. Its action would therefore be constant and we would have the same number of facets at all temperatures.

Hypothesis 5. Considering the Reduction in Facet Number to Be Due to an Inhibitor, Constant in Amount, but with a Rate Independent of the Temperature.—Under these conditions the inhibitor working through an increased time interval at the lower temperatures would produce a greater reducing effect and we would find more facets at the higher temperatures.

Hypothesis 6. Considering the Amount of Inhibitor to Be a Function of the Temperature, and that More of It Is Produced at High Temperatures than at Low.—Obviously this condition would explain the results obtained in bar, but it is merely restating the question in another form, as we should then have to explain why more inhibitor was produced at one temperature than at another.

Hypothesis 7. Considering the Decrease in Facet Number to Be Due to an Inhibitor the Temperature of Which Coefficient Differs from That of the Normal Facet-Producing Reaction.—Let N be the normal number of facets in the full eye. Let nt be the length of the period at T_1° during which facet rudiments are being produced. t is the length of the period at T_2° .

In full eye, at T_1° N facets are formed at a rate of $\frac{N}{n}$ per t for a period of nt ; at T_2° N facets are formed at a rate of N per t for a period of t .

In bar eye, N is reduced to Bx at T_1° , to By at T_2° .

Facets are formed then at the rate of $\frac{Bx}{n}$ per t at T_1° .

Facet number is reduced at the rate of $\frac{N}{n} - \frac{Bx}{n}$ per t at T_1° .

Facets are produced at the rate of By per t at T_2° .

Facet number is reduced at the rate of $N - By$ per t at T_2° . Then the rates of production and reduction have the following temperature coefficients.

$$\frac{N}{n} = \text{rate of production at } T_1^\circ$$

$$Q_{T_2} - T_1 = n$$

$$N = \text{rate of production at } T_2^\circ$$

When

$$\frac{N - Bx}{n} = \text{rate of reduction at } T_1^\circ$$

$$Q_{T_2} - T_1 = n$$

$$Bx = By$$

$$N - By = \text{rate of reduction at } T_2^\circ$$

$$Q_{T_2} - T_1 > n$$

$$Bx > By$$

$$Q_{T_2} - T_1 < n$$

$$Bx < By$$

In all the bar stocks, Bx is greater than By , where T_1 is the lower temperature. Hence the temperature coefficient for the reduction reaction in bar is greater than that of the production reaction. We can thus explain the difference in temperature relations between the full-eyed stocks and the bar-eyed stocks.

How can we explain the differences for the various bar-eyed stocks? From the above formulas it is obvious that the greater the difference between Bx and By the greater will be the difference between the Q_{10} for full and for bar. Bar is a changed condition which differs from the full in number of facets and also in the temperature coefficients of some of the reactions concerned in facet production. Is Ultra-bar a change in the same direction?

Ultra-bar effects a further decrease in the number of facets. But, as seen by the values of Bx and By , its temperature coefficient is really nearer that of the full than is that of the other bar stock. Ultra-bar is then not an increased condition of both these factors. The reversed change in the temperature coefficient may be a question of the concentration of the inhibitor.

Period during Which the Character of a Certain Structure Is Determined as Shown by the Temperature Effects.

Vernon² sums up the work of this nature on Lepidoptera. Dorfmeister concluded that the temperature had its greatest effect during the change from larva to pupa. According to Weismann, temperature acted at the beginning of the pupal period in *Vanessa prosa*. Merrifield concluded that the markings are chiefly affected during the early part of the pupal period, while coloring is affected during the penultimate pupal stage.

Hoge showed that exposure of the eggs to cold produced the greatest percentage of reduplication of legs in the imago of the fruit fly. Evidently the materials which determine the structure of the legs are differentiated in early embryonic development.

The bar eye factor comes into play after about three-fourths of the larval period is finished.

These last two cases are of interest in showing that some of the reactions which are involved in the differentiation of specific adult structures may occur at very early stages of immature life. Environmental stimuli must therefore be applied at definite periods in order to modify the organization of the adult.

The Direct Effect of Temperature upon the Mechanism of Inheritance.

Plough has shown that temperature has a definite effect on the percentage of crossing over between the hereditary materials of the second chromosome in *Drosophila*. He gets a maximum percentage at 13° and at 31° with a minimum from 22–27°. This curve Plough compares to the curve of Howell for the amount of contraction of the frog muscle at varying temperatures under constant stimulus. It is decidedly not a van't Hoff curve. Plough refers the phenomenon further to Lillie's results on the activation of the starfish eggs at various temperatures and concludes that temperature "probably causes some alteration in the physical basis of the egg."

The temperature effect on actual facet number seems to have nothing in common with the above results. If, however, we consider the per cent of increase or decrease per °C. some very interesting relations appear. Here, too, we find that the maximum change comes at the extreme high and low temperatures, with a minimum between, much as in the above reactions. With Plough, I leave the significance of the similarity of the cases to future research.

Individual Variation as Affected by Temperature.

In his book, Vernon³ makes the statement that "variability becomes steadily greater as the environment becomes more unfavorable."

² Vernon (1903), p. 241.

³ Vernon (1903), p. 218.

In an earlier article (1895), he remarks that variability reaches a maximum at 18–20°, the temperature most favorable for development.

The temperature experiments on bar eye offer data on this subject. As was pointed out previously, the data are not altogether consistent when any attempt to draw striking conclusions is made.

An examination of the data published by Vernon is even less satisfactory and warrants neither of the conclusions above ascribed to him.

The present study has value only as a preliminary to the subject of the effect of temperature on individual variation. The two following conclusions are suggested if not proved. (1) When measured in terms of the coefficient of variability, variability increases with temperatures. (2) When measured in terms of standard deviation, variability decreases with increase in temperature.

Considerations of the Straight Line Feature of Physiological Reaction Curves, and of the Exponential Curve for Facet Number.

Variability in Q_{10} .

Variability in the temperature coefficient, Q_{10} , occurs in practically all chemical reactions. The typical variation is a slight decrease as the temperature rises. Trautz and Volkmann give some interesting values for saponification reactions in which there is first a slight increase and then a steady decrease in Q_{10} with increase in temperature.

The variation of Q_{10} for chemical reactions is in no way comparable to those of enzymatic and vital reactions.⁴ In nearly every case the latter show a marked optimum. Obviously above the optimum Q_{10} becomes negative. As pointed out in this paper the values of Q_{10} for the rate of immature development vary from 27.31 at the 15–16° interval to – 2.41 at the 29–30° interval. These values are out of all proportion to the 2 to 3 requirements of van't Hoff's law.

This change in the value and sign of Q_{10} has been explained by Arrhenius and others as due to secondary factors. Two processes are involved; (1) the increase of activity of the enzyme, and (2) the destruction of the enzyme itself at the higher temperatures. The temperature, having a combative effect on the two processes, gives the appearance as it increases of checking the primary one when the end-results alone are considered.

Blackman accepts this sort of explanation for vital reactions. In the rate of assimilation by the leaves of the cherry laurel, he has ingeniously demonstrated the probability of the occurrence of increased rates above the optimum although these rates are not directly measurable.

Snyder has attributed the decrease in rate of physiological reactions at the higher temperatures to the differences in viscosity of protoplasm. This physical

⁴ Ernst has shown an optimum in catalytic action of colloidal platinum upon H_2O_2 .

phenomenon has a negative temperature coefficient. Experiments demonstrate a decrease in rate of nerve conduction with increase in viscosity at a constant temperature.

Balls maintains that the more rapid accumulation at higher temperatures of the waste products retards the primary reaction. While these products are formed at the lower temperatures, they are disposed of at a rate sufficient to prevent the checking of the primary reaction. At high temperatures they are formed more rapidly than they can be carried away. Their experimental removal, by dilution of the surrounding medium, raised the optimum of growth for the sore-shin fungus considerably.

Coagulation of proteins, which has been advanced as an explanation of death at the higher temperatures, might be suspected of producing a retardation in rate at the submaximal temperatures.

Straight Line Physiological Reaction Curves.

Most physiologists have given up van't Hoff's formula as too inaccurate to have any practical value. They have abundantly demonstrated the metabolic rate relations to be a linear rather than an exponential function of the temperature.

Loeb's Hypothesis of Secondary Factors.

Loeb has recently explained the straight line character of the rate curves to be due to the flattening out of an exponential curve by secondary factors. He shows that the "rate of life" of the imago of *Drosophila* may be plotted as an exponential curve, and that there is no falling off at the higher temperatures.

The criticism can be made, however, that an examination of his rates above 31° demonstrates clearly the presence of secondary factors that would tend to convert a straight line curve into an exponential curve.

A consideration of these various explanations of straight line curves and optima is now in order in light of the present work.

Explanations of the Straight Line Temperature-Rate Relations and Optima, Based on the Data of Facet Number and Developmental Rate in Drosophila.

In the bar-eyed mutant of *Drosophila*, two distinct reactions have been examined in regard to the effect of temperature upon them. One gives a typical straight line curve with a marked optimum at 29°. The other gives an exponential curve without decrease in rate at the upper temperatures. From 15–27° these two curves approximate each other, suggesting a close similarity in the primary nature of the two reactions throughout. Above 27° these two curves diverge. Secondary factors have entered to retard the rate in one, and to transform an exponential curve into a straight line.

It is quite obvious that for the facet reaction there is no "enzyme destruction," as there is no falling off in rate at the higher temperatures. The optimum in the metamorphic curve shows that we are in the range of temperature where such destruction would be expected. The secondary factors then are not to be located in the principle of enzyme destruction.

It is likewise as evident that changes in viscosity of the protoplasm cannot explain the differences observed in the two reactions since both occur simultaneously in the same material.

We may extend the same objections to such explanations as coagulation of proteins, physical state of protoplasm, and allied phenomena.

Balls' explanation of optima consisted in the more rapid accumulation of waste products at high temperatures. The by-products of metabolism retard the rate of the primary vital reactions. Their experimental removal raised the optimum decidedly, but did not carry it to the maximum temperature of growth as would be the case were this the only explanation.

Differential Temperature Coefficients as an Explanation of the Straight Line Feature of Physiological Reaction Curves.

The one idea of Balls that shows greater possibilities of development is that of differential temperature coefficients. Vital reactions are a series of complex processes in which both chemical and physical phenomena are represented. It is inconceivable that all these should have the same temperature coefficients. Vernon (1895) has demon-

strated the fact in the gross anatomy of the echinoderm larvæ. Laughlin has recently shown that the various phases of mitosis have markedly different temperature coefficients. Osterhout has pointed out the complications arising from complex systems, in which the various reactions have different Q_{10} values.

Differentiation and growth are of a necessity synchronized processes. It is at the higher temperatures that the effects of diverse temperature coefficients would be most noticeable. If one stage in development must await another, it is quite obvious that the whole general process would be slackened in speed. At extreme temperatures regulation would become impossible.

As has been shown, the reaction by which the number of facets is determined is of relatively short duration. It is not complicated by the processes of growth. It shows a true chemical temperature coefficient throughout.

Metamorphosis involves many long and interdependent processes. The separate reactions do not have the same temperature coefficients. This is evident from Hertwig's curves for a close sequence of stages in the development of the frog tadpole. A rapid reaction must await with its end-products the slower one, before further development can proceed. The higher the temperature the more erratic will be the separate temperature effects, and the slower becomes the total rate of development.

It seems reasonable to conclude with Loeb that the straight line feature of physiological reaction curves, together with the special feature of optima, is due to the flattening out of an exponential curve by secondary factors. These factors are not specific such as enzyme destruction, viscosity changes, protein coagulation, or accumulation of waste products, but are the normal results of a differential temperature effect on the separate phases of growth, differentiation, and development.

The Inherited Effects of Temperature.

Induction.

Woltereck working on the size of the head in *Daphnia*, Middleton on the fission rate in *Stylonychia*, and Sumner on the length of the feet and tail in mice found that measurable effects could be produced by temperature. Furthermore the effect showed itself in a less degree in subsequent generations, although the

causal factor, extreme temperature, had been removed. To this phenomenon Woltereck gave the name of "induction" or "pre-induction" according to the number of generations involved.

No such effect as this was noted in connection with facet number as investigated in Experiment 51. It is possible, however, that by continued existence, generation after generation, at a high or low temperature, such an effect might be obtained.

Temperature as a Causal Factor in the Production of Mutations.

Tower found among his potato beetles color variations that persisted through subsequent generations. Presumably, since they were reared at high temperatures, the cause of the mutation lay in this fact.

The present study shows marked discontinuous variations at neither high nor low temperature. Temperature in the present study is not a factor in the production of mutations.

SUMMARY.

Three strains of the bar-eyed mutant of *Drosophila melanogaster* Meig have been reared at constant temperatures over a range of 15–31°C.

The mean facet number in the bar-eyed mutant varies inversely with the temperature at which the larvæ develop.

The temperature coefficient (Q_{10}) is of the same order as that for chemical reactions.

The facet-temperature relations may be plotted as an exponential curve for temperatures from 15–31°.

The rate of development of the immature stages gives a straight line temperature curve between 15 and 29°. Beyond 29° the rate decreases again with a further rise in temperature.

The facet curve may be readily superimposed on the development curve between 15 and 27°.

The straight line feature of the development curve is probably due to the flattening out of an exponential curve by secondary factors.

Since both the straight line and the exponential curve appear simultaneously in the same living material, it is impractical to locate

the secondary factors in enzyme destruction, differences in viscosity, or in the physical state of colloids.

Differential temperature coefficients for the various separate processes involved in development furnish the best basis for an explanation of the straight line feature of the curve representing the effect of temperature on the rate of physiological processes.

Facet number in the full-eyed wild stock is not affected by temperature to a marked degree.

The mean facet number for fifteen full-eyed females raised at 27° is 859.06.

The mean facet number for the Low Selected Bar females at 27° is 55.13; for the Ultra-bar females at 27° it is 21.27.

A consistent sexual difference appears in all the bar stocks, the females having fewer facets. This relation may be expressed by the sex coefficient, the average value of which is 0.791.

The average observed difference in mean facet number for a difference of 1°C. in the environment in which the flies developed is 3.09 for the Ultra-bar stock and 14.01 for the Low Selected stock.

The average proportional differences in the mean for a difference of 1°C. are 9.22 per cent for Ultra-bar, and 14.51 for Low Selected.

The differences in the number of facets per °C. are greatest at the low and least at the high temperatures.

The difference in the number of facets per °C. varies with the mean.

The proportional differences in the mean per °C. are greatest at the lower (15–17.5°) and higher (29–31°) temperatures and least at the intermediate temperatures.

Temperature is a factor in determining facet number only during a relatively short period in larval development.

This effective period, at 27°, comes between the end of the 3rd and the end of the 4th day.

At 15°, this period is initiated at the end of 8 days following a 1st day at 27°.

At 27° this period is approximately 18 hours long. At 15° it is approximately 72 hours long.

The number of facets and the length of the immature stage (egg-larval-pupal) appear related when the whole of development is passed at one temperature.

That the number of facets is not dependent upon the length of the immature stage is shown by experiments in which only a part of development was passed at one temperature and the remainder at another.

Temperature affects the reaction determining the number of facets in approximately the same way that it affects the other developmental reactions, hence the apparent correlation between facet number and the length of the immature stage.

Variability as expressed by the coefficient of variability has a tendency to increase with temperature. Standard deviation, on the other hand, appears to decrease with rise in temperature.

Neither inheritance nor induction effects are exhibited by this material.

This study shows that environment may markedly affect the somatic expression of one Mendelian factor (bar eye), while it has no visible influence on another (white eye).

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE INACTIVATION OF PEPSIN SOLUTIONS.

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(Received for publication, February 14, 1920.)

One of the many factors which must be taken into consideration in any experiments with enzymes is the possible inactivation of the enzyme during the course of the reaction. This factor in the case of pepsin has been suggested by Sørensen¹ as the cause of the displacement of the optimum acidity for the digestion of protein to the acid side during the course of the digestion. He considers that the enzyme is more rapidly destroyed by the weak than by the strong acid. Arrhenius,² on the other hand, considers that the decrease in the rate of digestion on the acid side of the optimum hydrogen ion concentration for digestion is due to the more rapid destruction of the enzyme by the strong acid. If this explanation is correct the optimum phenomenon loses much of its significance and becomes a secondary characteristic of enzyme activity comparable to the optimum temperature. The possibility also arises that the peculiar falling off of the rate of digestion during the course of the reaction, at any hydrogen ion concentration, is also due to the destruction of the enzyme.

Several investigations³ have been made on the stability of pepsin in acid solutions from various points of view but the results are not at all concordant. Much of this variation in results is probably due to the failure to realize the importance of the hydrogen ion concentration rather than the total acid concentration.

¹ Sørensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1909, viii, 162. Sørensen's experiments were made at 52°. They are therefore not strictly comparable with the present results.

² Arrhenius, S., *Quantitative laws in biological chemistry*, London, 1915, 44.

³ Biernacki, E., *Z. Biol.*, 1891, xxviii, 49. Grober, J. A., *Arch. Exp. Path. u. Pharmacol.*, 1904, li, 103. Liebmann, P., and Johannesen, L., *Ugesk. Læger*, 1911, lxxiii, 902. Ramsay, C. F., *J. Am. Pharm. Assn.*, 1917, vi, 1047.

In the experiments considered in this paper the effect of the following variables on the inactivation of pepsin in solution has been studied: (1) the hydrogen ion concentration; (2) the anion of the acid; and (3) the purity of the enzyme solution.

The results of the experiments are given in Tables I and II and in Figs. 1 and 2. The figures in the tables are the relative amounts of active enzyme present in the solution after 24 or 48 hours. The total active enzyme present at the beginning of the experiment is taken as 10 units. The time required to cause a constant change

TABLE I.

Influence of the Purity of the Enzyme Solution on the Destruction of Pepsin at Various Hydrogen Ion Concentrations.

pH	Relative amount of pepsin per cc. of solution after 48 hrs. at 38°C.		
	0.25 per cent active pepsin.	2.5 per cent weak pepsin.	1.5 per cent weak pepsin in 3 per cent egg albumin solution.
6.2		1.0	
5.9			4.6
5.5	2.0	7.4	7.2
5.1	10.0		
4.7		8.4	8.2
4.0	10.0		7.9
3.6	9.0	6.8	7.2
2.0		6.3	7.0
1.2	8.0	6.0	6.8
0.6	7.8		

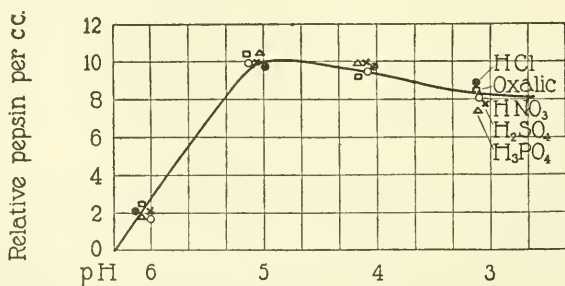


FIG. 1. Relative amount of active pepsin at different hydrogen ion concentrations with various acids after 24 hours at 38°C.

in the conductivity of an egg albumin solution under constant conditions is considered as inversely proportional to the amount of active enzyme present and was used as a measure of the enzyme concentration. The experiments with different enzyme solutions were not done at the same time and are not strictly comparable. The experiments with the various acids, however, are comparable.

It will be seen that in all the experiments the enzyme is most stable at a pH of about 5.0, irrespective of the anion of the acid and of the purity of the solution. Increasing the alkalinity of the solution causes a very great increase in the destruction of the enzyme. There is some indication that the impure solutions are inactivated more slowly under these conditions than the purer ones.

TABLE II.

Influence of Various Acids on the Destruction of Pepsin at Various Hydrogen Ion Concentrations.

pH	Relative amount of pepsin per cc. of solution containing the acids noted below after 24 hrs. at 38°C.				
	HNO ₃	H ₂ SO ₄	H ₃ PO ₄	Oxalic.	HCl
6.0-6.2	1.7	2.1	1.8	2.5	2.2
5.0-5.2	10.0	10.0	10.3	10.0	9.8
4.0-4.2	9.5	10.0	10.0	9.2	9.6
3.0-3.2	8.0	7.9	7.6	8.5	8.8

Increasing the acidity of the solution above pH 5.0 causes a very slow increase in the amount of pepsin destroyed, and the quantity inactivated is not influenced either by the purity of the solution or by the anion of the acid. It would seem necessary to conclude from the marked asymmetry of the curve for the destruction of the enzyme, as plotted against the hydrogen ion concentration, that the process of inactivation of the enzyme on the acid side of pH 5.0 differs from the process of inactivation on the alkaline side of pH 5.0.

Fig. 2 shows that the amount of pepsin remaining in solution after 24 hours at 38°C. is about the same throughout the range of acidity in which the enzyme is active. The rate of destruction of the enzyme therefore differs very little at a pH of 1.0 and a pH of 3.0. As is well known, the activity of the enzyme varies greatly within this range.

This is shown by Curve D, Fig. 2, which is taken from Sørensen's paper and which represents the rate of digestion of egg albumin by pepsin at various hydrogen ion concentrations. If the decline in the rate of digestion on the acid side of pH 2.0 was due to the increased destruction of the pepsin by the acid in greater concentration than this, the same drop should be noticed in Curves A, B, and C as in Curve D, since these curves represent the actual amount of destruction of the enzyme at the acid concentration in question. Fig. 2 shows that this is not the case. But little more enzyme was destroyed at a

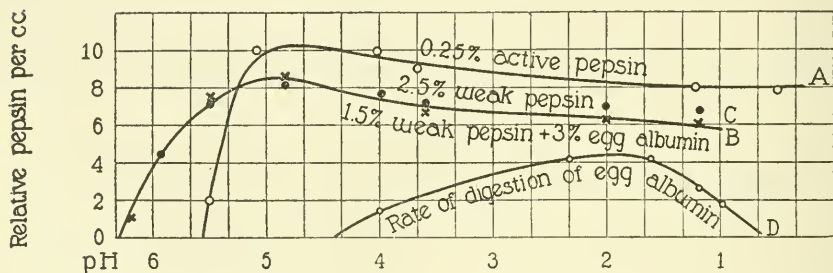


FIG. 2. Relative amount of active pepsin in various solutions at different hydrogen ion concentrations after 24 hours at 38°C.

pH of 1.0 than at a pH of 2.0 or 3.0. The rate of destruction is in any case much too slow to account for the rapid drop in the rate of the digestion curve. This drop is noticeable in the first few minutes of the reaction, while, as the figures show, only 10 to 20 per cent of the enzyme is destroyed in 48 hours at this hydrogen ion concentration.

The fact that the action of the acid on the enzyme is nearly the same across the whole range of hydrogen ion concentration in which the enzyme is active may be considered as indirect evidence that the optimum phenomenon is connected with changes in the substrate rather than in the enzyme. It is apparent from the figures that the enzyme is most stable at a pH of about 5.0; *i.e.*, the same as that for the isoelectric point of many proteins. There is no evidence, however, that pepsin is isoelectric at this point. A series of migration experiments made by the writer confirmed those of Michaelis and Davidsohn⁴ (except that the enzyme was never found to migrate to both

⁴ Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1910, xxviii, 1.

poles at the same pH) and gave a change in the direction of migration at about pH 3.0. There is no relation between this point and either the resistance of the enzyme to acid or the rate of its action on proteins. It is probable that this is not the isoelectric point of pepsin itself but that of a compound formed between pepsin and some other substance in the solution, since Peckelharing and Ringer⁵ found that very pure pepsin solutions showed no isoelectric point.

No evidence was found that the inactivation of the enzyme was reversible under the conditions of these experiments although many experiments were made with this point in view.⁶

The results show that digestion experiments with pepsin cannot be carried out at 38° for longer than 24 hours without being complicated by the fact that the enzyme concentration is lower at the end of the experiment than at the beginning. They also show that in experiments on the decomposition temperature it is necessary to consider the reaction of the medium.

The general effect of the hydrogen ion concentration on the stability of the enzyme resembles that described by Falk⁷ for lipase, and by Frankel⁸ for papain. In the case of papain, however, the influence of the reaction is reversed; *i.e.*, papain is more sensitive to acid than to alkali.

Experimental Procedure.

Pepsin Preparations Used.—Active: Fairchild's pepsin U. S. P. 1:19,500
Weak: Pepsin U. S. P. 1:3,000.

Hydrogen Ion Determinations.—All determinations were made by the E. M. F. method.

Determination of the Relative Amount of Pepsin in Solution.

The enzyme solution was made up as shown in the tables and placed in a water bath at $38 \pm 0.1^\circ\text{C}$. 5 cc. of the solution were pipetted out for analysis and 5 cc. of an acid solution added of such strength as to make the final acid concentration in each case equal

⁵ Peckelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

⁶ Tichomirow, N. P., *Z. physiol. Chem.*, 1908, lv, 107.

⁷ Falk, K. G., *J. Biol. Chem.*, 1917, xxxi, 97.

⁸ Frankel, E. M., *J. Biol. Chem.*, 1917, xxxi, 201.

to that of the solution containing the highest amount of acid. 1 cc. of this diluted solution was then added to a standard egg albumin solution and the time necessary to cause a 10 per cent change in the conductivity of the latter determined as described in a previous paper.⁹ The relative concentration of active pepsin was about the same at the beginning of each experiment. This quantity was taken as 10 in each case. Under the conditions of these experiments neither the products of the digestion of the egg albumin nor the inactivated pepsin interferes with the determination; *i.e.*, the reciprocal of the time to cause a given change is directly proportional to the total quantity of active pepsin present.

SUMMARY.

1. Pepsin in solution at 38°C. is most stable at a hydrogen ion concentration of about 10^{-5} (pH 5.0).

2. Increasing the hydrogen ion concentration above pH 5.0 causes a slow increase in the rate of destruction of pepsin.

3. Decreasing the hydrogen ion concentration below pH 5.0 causes a very rapid increase in the rate of destruction of the enzyme.

4. Neither the purity of the enzyme solution nor the anion of the acid used has any marked effect on the rate of destruction or on the zone of hydrogen ion concentration in which the enzyme is most stable.

5. The existence of an optimum range of hydrogen ion concentration for the digestion of proteins by pepsin cannot be explained by the destruction of the enzyme by either too weak or too strong acid.

⁹ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 113.

THE EFFECT OF THE CONCENTRATION OF ENZYME ON THE RATE OF DIGESTION OF PROTEINS BY PEPSIN.

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(Received for publication, February 16, 1920.)

The study of the kinetics of enzyme action has led almost invariably to results differing more or less from those predicted by the general laws of chemistry. It would be expected from the general theory of chemical reactions that enzyme reactions should conform to the law expressing the rate of a monomolecular reaction, accelerated by the presence of a catalyst. The rate of reaction, therefore, should be proportional to the concentration of the enzyme and of the substrate and should decrease with time as predicted by the monomolecular formula. This has been found to be true in rare instances^{1,2}; but in general the monomolecular formula does not hold for enzyme reactions. It has been found in many cases that the products of reaction interfere with the action of the enzyme. This would account for the divergence of the rate of reaction from that predicted by the monomolecular formula, since, owing to the action of the products, the concentration of the enzyme is changing during the course of the reaction, while the monomolecular formula takes account only of the changes in concentration of the substance decomposed. The rate of reaction of two solutions containing different amounts of enzyme, however, if compared during the same stage of the reaction, should be proportional to the quantity of enzyme, since any effect of the products should be the same in both solutions. It is found in many instances that this is not the case. Enzyme reactions diverge from the expected course of such reactions not only as regards the change in rate with the progress of the reaction, but also in regard to the relation between the rate and the concentration of substrate or enzyme.

¹ Euler, H., *Z. physiol. Chem.*, 1907, li, 213.

² Taylor, A. E., *J. Biol. Chem.*, 1906-07, ii, 87. Also Schmitz, H., *J. Gen. Physiol.*, 1919-20, ii, in press.

It was suggested by Brown³ that these divergences in the case of invertase were due to the fact that the enzyme formed an intermediate compound with the substrate; and several formulas⁴ which fit the experimental facts fairly well have been derived on this assumption. They all contain several arbitrary constants, however, and in the lack of any direct evidence in favor of the mechanism which they assume the agreement between calculated and observed values can hardly be considered conclusive. It is assumed in attempting to explain the mechanism of enzyme reactions from the point of view outlined above, that all the enzyme and all the substrate molecules present are equally able to take part in the reaction; in other words, that the active concentration and total concentration of enzyme (or substrate) are the same or directly proportional to each other. It is obvious that, if the active concentration of substrate or enzyme was not equal to the total concentration, the law of mass action would fail to hold if the total concentrations were used in formulas derived from this law, since the law itself states only that the rate of reaction is proportional to the active concentration of the reacting substances. It appears *a priori* quite possible that active enzyme or substrate molecules may exist in solution in equilibrium with other molecules which do not take part in the reaction. The concentration of active enzyme molecules (in the sense of the law of mass action, *i.e.* those which take part in the reaction) would then be some other function of the total concentration and would not be directly proportional to it. The rate of reaction would then also be found to vary as some other function of the total enzyme concentration and not in direct proportion to it. An exactly analogous case is well known in general chemistry; namely, acid hydrolysis.⁵ The hydrogen ion is the active part

³ Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

⁴ Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 146. These authors review the various other formulas proposed. See also Moore, B., in Hill, L., *Recent advances in physiology and biochemistry*, New York and London, 1906, 43.

⁵ For a general discussion of this question see Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 166, 402, 650. Stieglitz's experiments were made on the hydrolysis of esters. These solutions can hardly be considered heterogeneous and yet show the same divergences from the simple mass action law as do enzyme reactions. This question will be discussed more fully in a subsequent paper.

of the molecule and the rate of reaction therefore varies directly with the hydrogen ion concentration and not with the total acid concentration. In sufficiently dilute solutions the two of course become practically identical since the acid is then completely dissociated. It will be shown in the succeeding part of this paper that pepsin solutions obey the same laws as weak acid solutions in regard to the relation between the total concentration and the rate of hydrolysis; and that the divergence from the law of mass action is not due to any peculiarity of the enzyme reaction itself, but to the fact that the active enzyme concentration is not always directly proportional to the total enzyme concentration.

Experimental Procedure and Results of the Present Investigation.

In a former paper⁶ a method was described for determining the rate of pepsin digestion by means of changes in the conductivity of an egg albumin solution to which the pepsin had been added. From these results the time necessary to cause a given change in the conductivity of the solution was determined by graphic interpolation. In the experiments reported in this paper the time in hours necessary to cause the first 10 per cent change was taken as the standard. The reciprocal of this time then $\left(\frac{1}{T \text{ hours}}\right)$ is proportional to the mean rate of digestion for the first 10 per cent of the reaction. For convenience this value will be spoken of as the amount of "active pepsin." The volume noted in the tables is considered in every case as the number of cc. of diluted enzyme solution containing 1 cc. of the original enzyme solution. It is therefore a measure of the dilution of the pepsin before adding to the egg albumin solution. Since 1 cc. of this diluted solution was added to 25 cc. of egg albumin in order to make a determination, the concentration of the pepsin during the actual digestion was $\frac{1}{26}$ of that shown in Tables I, II, III, and V. The conductivity and pH of all solutions were kept equal as nearly as possible. It was pointed out that this change in conductivity did not exactly parallel the change in amino nitrogen of the solution, and so cannot be considered as representing the true course

⁶ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 113.

of the reaction. If the amount of egg albumin and all other factors except the amount of pepsin are kept equal, however, the time necessary to cause a given change may be considered as a definite measure of the rate of reaction, which is all that is necessary for the present purpose.

It was stated⁶ that the rate of reaction (*i.e.* the reciprocal of the time to cause a given change) was directly proportional to the concentration of enzyme solution, and that any products of reaction present in the enzyme solution did not interfere with the reaction. Both statements were true as regards the pepsin solutions used in the experiments reported. It was found, however, that some pepsin solutions did not obey this law. The rate of digestion, instead of being directly proportional to the enzyme concentration, increased much more slowly. The same phenomena have been observed by Bayliss in the case of trypsin⁷ and invertase,⁸ and have frequently been observed in enzyme reactions. It has formed one of the arguments for the conception that the enzyme combines with the substrate according to the adsorption formula.^{8,9}

Table I is a summary of an experiment illustrating this point. The results are shown graphically in Fig. 1. It is obvious that the value of *ET* (total pepsin concentration \times the time necessary to cause 10 per cent of the total change in conductivity) is constant for low concentrations but increases in higher concentrations. (If the rate of reaction is directly proportional to the enzyme concentration, the value of *ET* must of course be constant.) The calculated figures were obtained by a formula considered below. The key to this behavior is given by the results of the experiments shown in Table II. In this experiment 2.5 cc. of an active pepsin preparation were diluted to 10 cc., A, with HCL (pH 2.0) and, B, with a solution of "peptone"¹⁰ prepared by the digestion of egg albumin by a very small amount of pepsin (but containing no active pepsin). Solutions A and B were

⁷ Bayliss, W. M., *Arch. Sc. Biol.*, 1904, ii, suppl., 261.

⁸ Bayliss, W. M., *Proc. Roy. Soc. London, Series B*, 1911-12, lxxxiv, 90. Duclaux, E., *Chimie Biologique*, Paris, 1883.

⁹ Bayliss, W. M., *The nature of enzyme action*, Monograph on Biochemistry, London, New York, Bombay, and Calcutta, 3rd edition, 1914.

¹⁰ The word peptone is used in this paper as a general term for substances with which pepsin combines in solution, but does not hydrolyze.

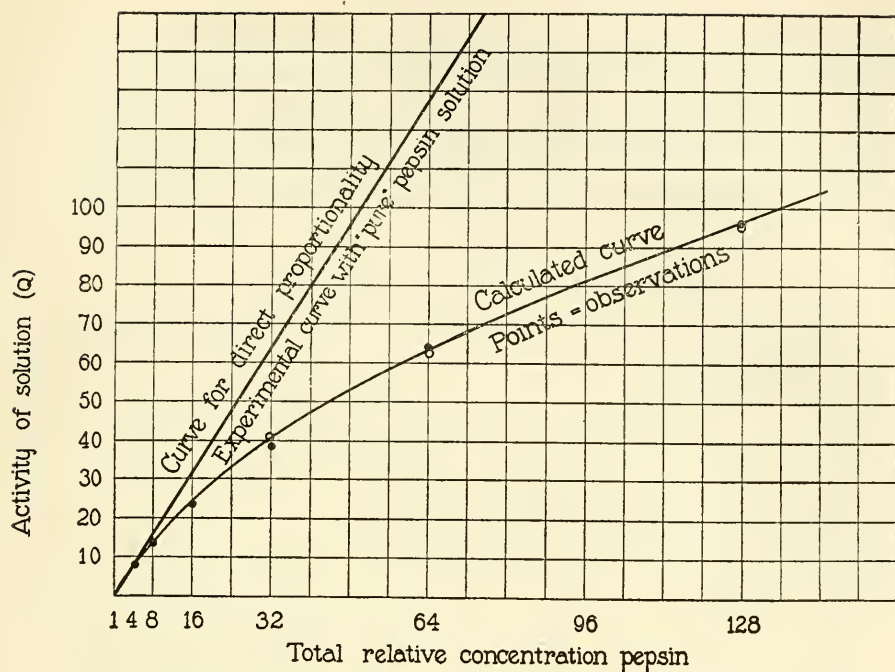


FIG. 1. Curves showing pepsin concentration and rate of digestion (*cf.* Table I).

TABLE I.

Enzyme Concentration and Rate of Digestion.

Pepsin solution. 10 per cent solution of Gr bler's pepsin in HCl, pH 2.0.

$$K = 7.2 \quad d = \frac{30}{v}$$

V = volume containing 1 cc. of original pepsin solution.	E = total pepsin per cc.	$Q = \frac{1}{T} = \text{active pepsin per cc.}$					ET
		Observed.				Calculated.	
		1	2	3	Average.		
1	26.9	9.1	9.7	10.0	9.6	9.7	269
2	13.44	6.25	6.30	6.67	6.39	6.40	206
4	6.72	4.17	3.70	3.57	3.81	4.05	175
8	3.36	2.38	2.50	2.17	2.35	2.42	145
16	1.68	1.39	1.43	1.35	1.39	1.38	120
32	0.84	0.83	0.80	0.78	0.80	0.77	106
64	0.42	0.41	0.40	0.39	0.40	0.40	100
128	[0.21]	0.22	0.20	0.20	0.21	0.20	100

then diluted as shown in the table with HCl while Solution C was diluted with Solution B in which the pepsin had been inactivated by making the solution alkaline for 10 minutes. With Solutions A and C the product of the time into the amount of pepsin present is constant as required by the law of mass action, while in Solution B the value

TABLE II.

Effect of Addition of Peptone to Pepsin Solutions.

Solution A. 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + HCl, pH 2.0.

Solution B. 2.5 cc. of 2 per cent active pepsin diluted to 10 cc. + 1 per cent peptone solution, pH 2.0.

Solutions A and B then diluted as noted + HCl. Pepsin determined in 1 cc.

Solution C. Same as B except diluted with inactivated B, instead of HCl.

V = volume containing 1 cc. of original pepsin solution.	E = relative concentration of total pepsin taken.	Time for 10 per cent change in conductivity of 25 cc. of egg albumin + 1 cc. of solution. $T = \text{hrs.} \times (10^2)$.			ET for solution.		
		A	B	C	A	B	C
1.0	100	20	28	21	20.0	28.0	21.0
1.5	66	31	34	29	20.5	22.5	19.1
2.0	50	40	39	41	20.0	19.5	20.5
4.0	25	83	79	81	20.7	19.8	20.3
8.0	12.5	170	162	178	21.2	20.3	22.2

of the product decreases with increasing dilution until it becomes equal to that value obtained from Solutions A and B, and then remains constant.¹¹ The results are plotted in Fig. 2. The straight line represents direct proportionality.

The solutions were made up to contain the same total concentration of pepsin and in the higher dilutions show the same degree of activity. It seems, therefore, that the divergence of Solution B from the regular law must be due to the fact that the peptone combines with the pep-

¹¹ This experiment is probably the explanation of the conflicting results obtained by Bayliss⁸ and Nelson and Vosburgh (Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790) in connection with the action of invertase. The activity of the solution of invertase used by Bayliss was not proportional to its concentration whereas the activity of that used by Nelson and Vosburgh was directly proportional.

sin to form a rather highly dissociated compound and that the pepsin so combined is inactive. The concentration of active pepsin would therefore be decreased by the peptone and the decrease would be greater in concentrated than in dilute solution. This hypothesis also accounts for the results of Experiment C in which the solution is diluted with an inactivated portion of the same solution. If the inactivated pepsin enters into equilibrium in the same way as the

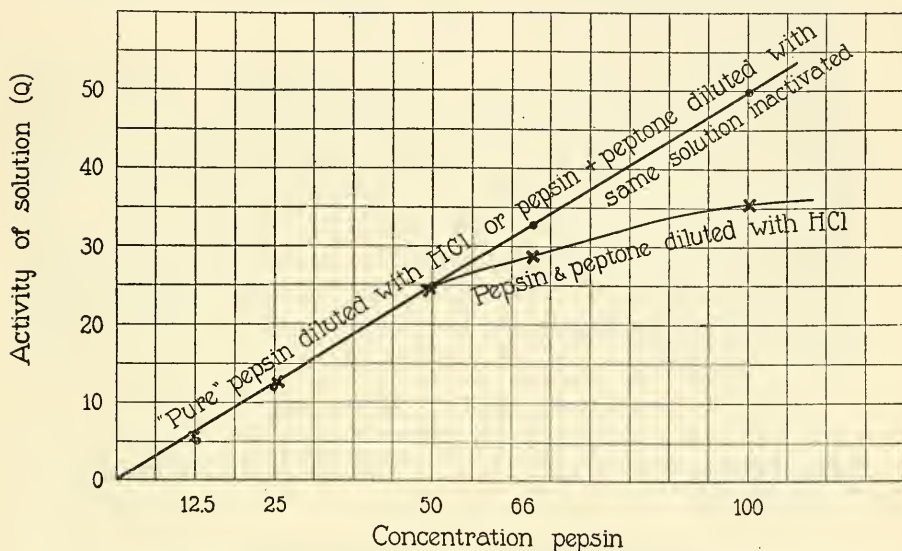
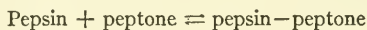


FIG. 2. Curves showing effect of peptone on activity of diluted pepsin solutions (cf. Table II).

active pepsin, the concentration of active pepsin in a solution, diluted with an inactivated portion, should decrease in direct proportion to the total concentration. The experiment shows that this is the case. (This question will be taken up more fully later.) The results of this experiment show also that in order to determine the total amount of pepsin present in solution it is necessary to use a dilution such that the rate of digestion is directly proportional to the amount of enzyme solution taken. If this is done the value for the total amount of enzyme, found at dilutions where this value has become constant, is an experimental determination of the total amount of enzyme present, expressed, however, in arbitrary units.

The effect of the peptone in Solution B might be qualitatively explained by the hypothesis that the peptone in the solution combines with the substrate and so reduces the concentration of active substrate molecules, thereby causing the enzyme to become "saturated" with substrate. This explanation, however, fails to explain the results of Experiment C since the same concentration of peptone is present here as in Solution B and yet in this experiment the rate *is* proportional to the amount of enzyme taken.

According to the hypotheses outlined above, the rate of digestion is always directly proportional to the concentration of active pepsin; and the apparent divergence from this relation is due to the fact that the peptone combines with the pepsin and so renders it inactive. The total concentration of enzyme and the active concentration are then no longer equal nor directly proportional; and since the rate is proportional to the active concentration, it is not proportional to the total concentration. It is also assumed that the pepsin and peptone combine according to the law of mass action. This reaction may be considered to take place as follows:



and if the reaction obeys the law of mass action the following equation must hold.

$$\frac{\text{Concentration pepsin} \times \text{concentration peptone}}{\text{Concentration pepsin-peptone}} = K \quad (1)$$

or

$$\frac{Q \cdot (d - (E - Q) + x)}{E - Q} = K \quad (2)$$

where E is the total enzyme concentration, Q is the concentration of active (uncombined) pepsin, d is the concentration of peptone present at the beginning of the reaction, and x is the amount of peptone formed during the course of the reaction at the time t . K is the equilibrium constant expressed in arbitrary units since it contains the unit of measurement used. (For the sake of simplicity only the case is considered in which the substance combined with the pepsin at the beginning of the reaction is the same as that formed during the

digestion.) The value of Q then (the active pepsin concentration) at any moment of the reaction would be that defined by equation (2) or

$$Q = -\frac{d - E + x + K}{2} + \sqrt{\left(\frac{d - E + x + K}{2}\right)^2 + KE} \quad (3)$$

The differential equation for the whole process¹² would then be

$$\frac{dx}{dt} = kQ(A - x) \quad (4)$$

in which Q has the value expressed in equation (3), and A is the active concentration of substrate present at the beginning of the reaction. (It seems quite probable that the active substrate concentration is related to the total substrate concentration in the same way as the active and total enzyme concentrations are related. This question will be discussed later. For the present it is assumed that the rate is proportional to the substrate concentration. At low dilutions of substrate this is an experimental fact.) If this value for Q is substituted in equation (4) it becomes too unwieldy in the integral form to use conveniently. The equation may be tested in the differential form, however, by choosing a small constant value for Δx (taken as 10 per cent of the total change in these experiments) and determining Δt experimentally. The reciprocal of the time necessary to cause the change will then be proportional to the mean rate of reaction during the first 10 per cent of the hydrolysis. This rate is of course decreasing constantly due (1) to the decrease in substrate concentration, and (2) to the decrease in the concentration of active pepsin since some pepsin is removed by combination with the products of reaction. The relative decrease in Q due to (1) is the same in every case and cancels out in comparative experiments, such as are considered here, since the total substrate concentration is kept the same in every experiment. The relative decrease in rate (Q) due to (2), however (as may be seen from equation (2)), will not always be the same but will depend to some extent on the relative values of E , d , and x . It will be shown later that a 5 per cent egg albumin solution when completely digested contains about 10 units of peptone (Table III). The

¹² Neglecting any effect of the reverse reaction.

value of x therefore in the first 10 per cent of the reaction will increase from 0 to 1.0. The percentage decrease in Q (the concentration of active pepsin) will depend to some extent on the concentration of peptone (d) present at the beginning of the reaction. That this is actually so is shown by the fact that the relative rates of digestion of two solutions containing the same amount of pepsin but very

TABLE III.

Enzyme Concentration and Rate of Digestion.

Pepsin solution. 1 per cent active pepsin + 10 per cent egg albumin, pH 2.0.
Digested 24 hrs. at 37°C. Diluted as below + HCl, pH 2.0.

$$K = 8.5 \quad d = \frac{19.2}{v}$$

V = volume containing 1 cc. of pepsin solution.	E = total pepsin.	$Q = \frac{1}{T} = \text{active pepsin.}$			
		Observed.			Calculated.
		1	2	Average.	
1.0	10.95	4.35	4.24	4.29	4.40
1.18	9.32	4.17	4.05	4.11	4.08
1.43	7.66	3.84	3.57	3.70	3.71
1.66	6.58	3.45	3.50	3.47	3.39
2.0	5.48	3.03	2.98	3.00	3.06
2.5	4.39	2.94	2.78	2.86	2.67
3.33	3.28	2.17	2.38	2.27	2.17
5.0	2.18	1.75	1.50	1.62	1.61
10.0	1.09	1.0	0.98	0.99	1.05
20.0	0.54	0.57	0.52	0.55	0.52

different amounts of peptone vary, depending on what stage of the reaction is compared. This is due to the fact that the rate of digestion of the solution containing the peptone decreases more slowly than that of the solution containing no peptone. This is in agreement with the formula. The differences in the percentage decrease in the rates of digestion of two solutions during the first 10 per cent hydrolysis, due to variations in the relative values of E and d , were found to be too small to effect the results within the range of values of E and d used in these experiments. The relative mean rate for the first 10 per cent hydrolysis may therefore be considered proportional

to the amount of active pepsin present at the beginning of the reaction; *i.e.*,

$$\text{Rate} = \frac{1}{T} = Q = -\frac{d - E + K}{2} + \sqrt{\left(\frac{d - E + K}{2}\right)^2 + KE}$$

where T is the time in hours necessary to complete the first 10 per cent of digestion. This equation may be tested experimentally by testing the constancy of K for various values of E and d or, better, by comparing calculated and observed values of $\frac{1}{T}$ since small experimental errors cause very large changes in the value of K .

The results of such a series of experiments have been given in Table I. Table III contains the results of a similar experiment in which the pepsin solution was prepared by adding 10 per cent of egg albumin to an active pepsin solution and allowing digestion to be completed at a temperature of 38°C. The solution was then diluted as shown in Table III. As was the case in Experiment 1, the rate of digestion is not directly proportional to the total enzyme concentration. It will be seen that in both Tables I and III the agreement between calculated and observed values is within the experimental error. The figure for E , the total enzyme present, is determined directly from the experiments in high dilution when the value of ET has become constant. It was shown in Experiment 2 that the value for E obtained in this way was really proportional to the total amount of enzyme present. The value for d , the amount of peptone present at the beginning of the reaction, is determined from the figures themselves and therefore must be considered as a second arbitrary constant. This fact, of course, detracts considerably from the significance to be attached to the agreement between the observed and calculated values. It will be shown below, however, that under certain conditions the formula may be still further simplified so as to contain one constant and that it is still found to hold.

Table IV contains a summary of an experiment in which the total concentration of peptone was kept the same and the concentration of pepsin increased. The results are shown graphically in Fig. 3. The values for E , the total pepsin present in the solution of Gr  bler's pepsin, K , the equilibrium constant, and d , the concentration of pep-

tone originally present, were taken from Table I. The value for E in Solution B was determined by a separate experiment. It will be seen that the total amount of active pepsin found in the solution is not equal to the sum of the amount of active pepsin added plus the amount of active pepsin already present. This shows that the pepsin

TABLE IV.

Addition of Active Pepsin Solution to Solution of Gr bler's Pepsin.

Solution A. 10 per cent Gr bler's pepsin, pH 2.0.

Solution B. 3 per cent active pepsin, $E = 4.2$.

$K = 7.2$, $d = 3.0$, E (in Solution A) = 2.69 (Table I). 1 cc. of Solution A + noted cc. of B made up to 10 cc. $K = 7.2$.

Volume of Solution B added.	Units of active pepsin added per cc.	Units of active pepsin in Solution A per cc.	$Q = \frac{1}{T} = \text{total units of active pepsin.}$	
			Found.	Calculated.
cc.				
0	0	[2.08]	2.08	2.03
1	0.42	2.08	2.32	2.27
2	0.84	2.08	2.83	2.71
3	1.26	2.08	3.12	3.05
5	2.10	2.08	3.84	3.77
7	2.94	2.08	4.44	4.50
9	3.78	2.08	5.20	5.26

is in equilibrium with the substance that inhibits its action. The fact that the calculated values agree with those found by experiment shows that the equilibrium obeys the law of mass action since the calculated figures are obtained by means of this law.

The Effect of Inactivated Pepsin on the Equilibrium.

The results of Experiment 2 show that if a solution of pepsin (A) containing peptone is diluted with acid, the activity of the resulting solution *is not* directly proportional to the concentration of A. If the same solution is diluted with a portion of itself in which the pepsin has been inactivated with alkali, the activity of the resulting solution *is* directly proportional to the concentration of A. This is the result predicted if it is assumed that the inactive pepsin enters into the equilibrium (*i.e.* combines with the peptone) to the same extent as

the active pepsin. Table V summarizes the result of an experiment similar to Experiment 2 but covering a wider range. In this experiment an impure solution of pepsin (the same as used in Experiment 1) was diluted, A, with acid of the same hydrogen ion concentration, and, B, with the same solution which had been previously inactivated by alkali and then brought back to the same pH as the original. The

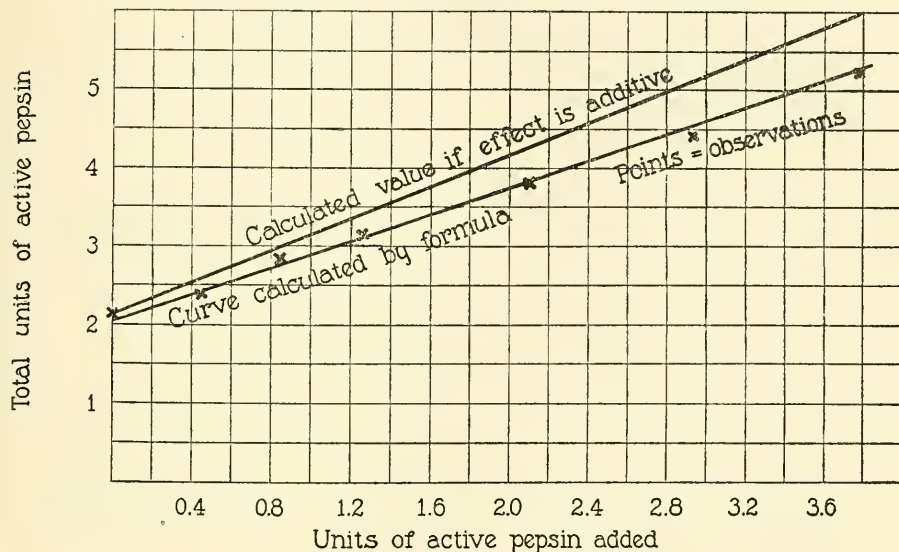


FIG. 3. Curves showing effect of addition of "pure" pepsin to pepsin solution containing peptone (*cf.* Table IV).

activity of the solution diluted with acid is not directly proportional to its concentration. When the same solution is diluted with an inactivated portion of itself, the activity of the resultant solution is directly proportional to its concentration. The figures show again that the result is predicted quantitatively by the hypothesis. Pepsin inactivated by alkali therefore retains the ability to combine with peptone exactly as does the active pepsin. It has, however, lost the power to hydrolyze protein. A very similar phenomenon is known in immunology—the so called toxoids; *i.e.*, toxins which are no longer injurious but are able to bind antibody in the same way as true toxin.

If the pepsin is inactivated by boiling instead of by treatment with alkali the results become irregular and do not agree with the hypothesis that the inactivated pepsin either does or does not enter into the equilibrium.¹³ In order to predict them quantitatively it becomes necessary to assume that either the equilibrium constant is changed

TABLE V.

Influence of Inactivated Pepsin on Equilibrium.

10 per cent Gr bler's pepsin diluted as noted with, A, HCl, pH 2.0. B, with same solution inactivated by alkali.

V = volume containing 1 cc. of original pepsin solution.	$Q = \frac{1}{T}$ = active pepsin observed in solution.		Calculated.	
	A	B	If inactive pepsin enters equilibrium.	If inactive pepsin does not enter equilibrium.
1	9.6	9.5	9.6	9.6
2	6.39	5.0	4.8	3.6
4	3.81	2.48	2.40	1.4
8	2.35	1.33	1.20	0.8
16	1.39	0.70	0.60	0.4

or that some of the peptone also is destroyed. In any case boiling causes a different change in the properties of a pepsin solution from inactivation with alkali.

Effect of Adding Increasing Amounts of Peptone to Pepsin Solutions.

It is possible to test further the hypothesis outlined in this paper by noting the effect of adding different amounts of peptone to a constant quantity of pepsin and comparing the observed and calculated activity of the resultant solution. If, as assumed in the hypothesis, the pepsin combines with the peptone to form a dissociated compound, the effect of adding successive equal amounts of peptone to a constant quantity of pepsin should not result in a constant decrease in activity of the solution for each unit of peptone added. The first unit of

¹³ An apparently similar phenomenon was noticed by Bayliss⁷ in his experiments with trypsin.

TABLE VI.

Effect of Adding Increasing Amounts of Peptone to Pepsin Solution.

0.5 cc. of 5 per cent active pepsin solution + noted cc. of peptone solution (from digested egg albumin) and made up to 10 cc. $K = 8.5$ (Table III), $E = 3.16$, $d = 0.85$ per cc. of peptone solution.

Peptone solution. cc.	$d =$ units of peptone added.	$Q = \frac{1}{T} =$ units of active pepsin.			Units of combined pepsin per unit of peptone added. Observed.	Qd
		Observed.	Average.	Calculated.		
0	0	3.03	3.16			
		3.14				
		3.33				
1	0.85	2.86	2.88	2.92	0.33	2.4
		2.86				
		2.94				
2	1.7	2.70	2.75	2.75	0.24	4.6
		2.70				
		2.86				
4	3.4	2.38	2.42	2.42	0.22	8.2
		2.40				
		2.50				
6	5.1	2.08	2.03	2.13	0.22	10.0
		2.04				
		1.96				
8	6.8	1.67	1.81	1.91	0.20	12.0
		1.85				
		1.92				

peptone added should have a greater effect than the second, the second a greater than the third, and so on; the relative decrease of the effect depending on the value of the equilibrium constant. Table VI and Fig. 4 give the result of an experiment carried out in this way. It will be seen that the effect of adding increasing units of peptone agrees very well with the calculated values. The compound pepsin-peptone is widely dissociated at this dilution inasmuch as with a total concentration of 0.85 units of peptone and 3.16 units of pepsin only 0.28 units are combined. This fact is shown graphically in

Fig. 4 where the straight line represents the concentration of active pepsin which would be present if the combination was complete. Table VI also shows that the first unit of peptone inactivates more pepsin than the second, etc. This phenomenon is also common in immunology and is known as Ehrlich's phenomenon. As Arrhenius¹⁴ has pointed out it is a general property of any equilibrium system.

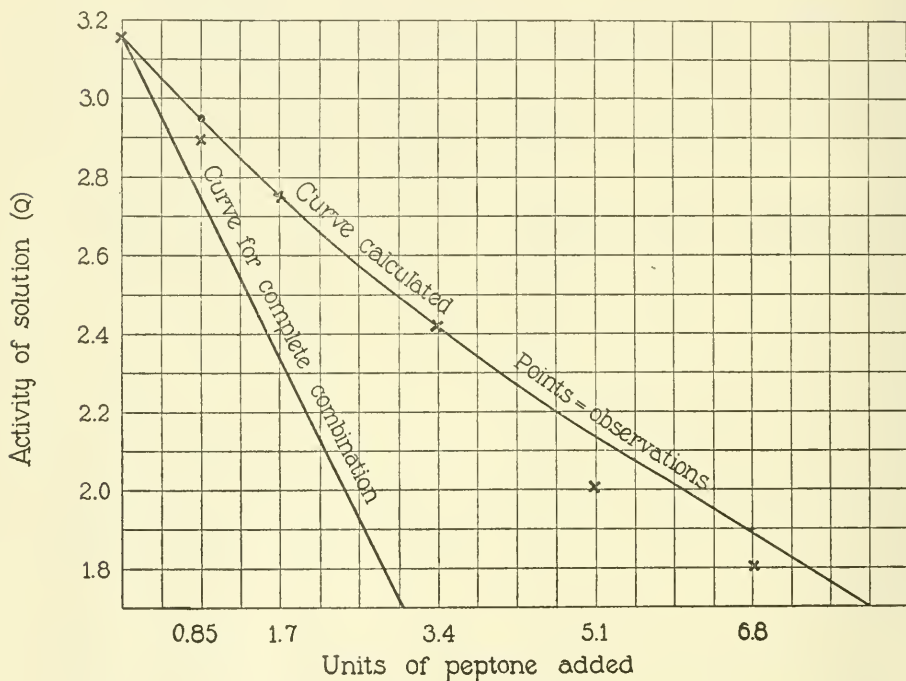


FIG. 4. Curves showing effect of adding increasing amounts of peptone to pepsin solutions (cf. Table VI).

In several other respects the action of pepsin on an egg albumin solution is more or less analogous to the action of toxin on an organism. In a sense the pepsin may be said to make the egg albumin solution immune to pepsin. That is, if a small amount of pepsin is allowed to act for a long time on a large quantity of albumin it will at first digest it very rapidly and the rate of digestion will be proportional to the amount of pepsin added. The rate of digestion decreases rapidly,

¹⁴ Arrhenius, S., *Ergebn. Physiol.*, 1908, vii, 480.

however, and finally becomes almost negligible in spite of the fact that there is still a large amount of egg albumin in solution and that the pepsin still retains its activity (as may be demonstrated by diluting the solution, after which digestion will continue). The addition of a further amount of pepsin to the solution will now have little or no effect. The albumin solution is "immune" to the pepsin. This is due to the fact that a small amount of pepsin can cause the production of a very large amount of peptone. Each unit of peptone produced decreases the amount of free pepsin somewhat; but as may be seen from equation (2) it would require an infinite concentration of peptone (d) to reduce the concentration of free pepsin (Q) to 0. Practically, the reaction stops owing to the destruction of the pepsin.¹⁵

Referring again to Table VI, it will be noted that Qd , the product of the concentration of active enzyme into the concentration of peptone, approaches a constant value as d increases. In other words the concentration of active enzyme becomes nearly inversely proportional to the concentration of peptone, when the latter is present in great excess. This is a well known property of mass action equilibria and follows from the formula, as may be seen below. The formula used in this connection is

$$\frac{Q \cdot [d - (E - Q)]}{E - Q} = K$$

where Q is the concentration of active (free) enzyme, $[d - (E - Q)]$ the concentration of free peptone, and $E - Q$ the concentration of combined pepsin or peptone. It is obvious that as d increases Q must decrease so that the value of the term $E - Q$ approaches the constant value E . When d becomes very large compared with E the term $d - (E - Q)$ will not differ significantly from d . The equation may then be written

$$Q = \frac{KE}{d}$$

d in this equation represents the amount of peptone present at the beginning of the reaction. If the equation is to hold throughout the reaction the concentration of peptone will be represented by $d + x$.

¹⁵ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 465.

If the simplest case is considered in which there is no peptone present at the beginning of the reaction the concentration of peptone at any time is x (since d is 0) and the formula becomes

$$Q = \frac{KE}{x}$$

This value for Q may now be substituted in equation (4) which becomes

$$\frac{dx}{dt} = \frac{KE(A-x)}{x} \quad (5)$$

in which A is the concentration of substrate at the beginning of the reaction and x is the amount of substrate decomposed (or of peptone formed) at the time T . For the first part of the reaction the value of $(A-x)$ will not differ much from the value of A and the equation may be still further simplified to

$$\frac{dx}{dt} = \frac{KEA}{x}$$

which states that the rate of digestion at any moment is directly proportional to the enzyme concentration and the substrate concentration, and inversely proportional to the amount of substrate decomposed. K in this equation is a new constant equal to the product of k , the velocity constant, and K (equation (4)), the equilibrium constant. This equation, as has been pointed out by Arrhenius,¹⁶ is the differential form of Schütz's¹⁷ rule, since on integration it becomes

$$TKEA = x^2 \quad \text{or} \quad x = K \sqrt{TEA}$$

That is x , the quantity of peptone formed, is proportional to the square root of the time, the concentration of pepsin, and the con-

¹⁶ Arrhenius, S., *Medd. Kong. vetsakad. Nobelinst.*, 1908, i. An equation similar to this but containing $x^{\frac{1}{2}}$ was found by Bodenstein and Fink (Bodenstein, M., and Fink, C. G., *Z. physik. Chem.*, 1907, lx, 1) to represent the rate of oxidation of SO_2 in the presence of platinum. Dernby, K. G., *Z. physiol. Chem.*, 1914, lxxxix, 425.

¹⁷ Schütz, E., *Z. physiol. Chem.*, 1885, ix, 577.

centration of substrate. It follows from this that if two solutions are compared, each containing the same quantity of substrate, and allowed to digest the same length of time, but with varying concentrations of enzyme, the amount of substrate digested will be proportional to the square root of the enzyme concentration. This is the usual form of Schütz's rule.

It will be remembered that in the derivation of this equation two simplifying assumptions were made: (1) that x , the concentration of peptone, is large compared to Q , the concentration of active pepsin; and (2) that the quantity of substrate present remains relatively constant. The first condition is fulfilled as soon as the digestion has progressed more than a few per cent, provided the original concentration of pepsin is small compared to the concentration of albumin. The second condition, on the other hand, fails to hold after more than 30 or 40 per cent of the substrate is digested. It can be predicted then that Schütz's rule will not hold during the first few minutes of the reaction, or at the end of the reaction, or if the enzyme concentration is too high. As is well known, this is exactly the result obtained by experiment (*cf.* Arrhenius¹⁶).

The failure of the rule to hold during the first part of the digestion is due to the fact that x at this time is not large compared with Q and hence the relative change in Q is not inversely proportional to the change in x (as assumed in the derivation of the equation) but is much slower as demanded by equation (2). In order to express the fact correctly for the first part of the reaction, then, it would be necessary to substitute for Q in equation (4) the value of Q as defined by equation (3). As has been previously stated, this expression is too unwieldy to handle conveniently. The discrepancy due to changes in the substrate concentration, however, may be corrected very simply if the rate of digestion is directly proportional to the concentration of substrate when the concentration of the latter is low. Experiment shows that this is actually the case. (The effect of the substrate concentration is at present under investigation.) The active concentration of substrate at any moment then will be $A - x$, where A is the original total concentration of substrate and x is the amount transformed. This has already been done in equation (5)

$$\frac{dx}{dt} = \frac{KE(A-x)}{x}$$

which on integration becomes

$$\frac{A \ln \frac{A}{A-x} - x}{ET} = K \quad (6)$$

If the foregoing hypothesis correctly expresses the mechanism of the reaction, the results calculated from Schütz's rule and equation (6) should agree with the experimental results as soon as x has reached a value ten or fifteen times as large as the quantity of active pepsin present. Before x has reached such a value, the results calculated from equation (6) or Schütz's rule, using the values of K at which they are constant, will be higher than those found by experiment. That is, the value of K in Schütz's rule or equation (6) increases for the first 10 or 20 per cent of the total digestion. As was pointed out above, this discrepancy is due to the fact that the formulas are derived on the assumption that the relative change in the pepsin concentration is inversely proportional to the change in the peptone concentration, a condition which does not hold until the peptone is present in large excess. After this point is reached both equations should correctly represent the course of the reaction until the changes in substrate become large. After this change in substrate concentration becomes significant Schütz's rule will no longer hold since there is no term in it that provides for the change in substrate concentration.¹⁸ Equation (6) should hold (*i.e.* give a constant value for K) until the end of the reaction, since this equation takes account of the changes in substrate concentration. Table VII and Fig. 5 give the results of an experiment in which the rate of hydrolysis of an egg

¹⁸ If the substrate concentration is high (*i.e.* more than 1 to 2 per cent) Schütz's rule will be found to hold throughout the greater part of the reaction. This is due to the fact that the rate of digestion in concentrated solutions is nearly independent of the substrate concentration. The falling off in the rate of reaction is therefore almost entirely due to the changes in the pepsin concentration. This change is correctly expressed by Schütz's rule. In high concentration of albumin Schütz's rule therefore fits better than Arrhenius' equation.

albumin solution has been followed by means of the conductivity. The total change was $\frac{1}{170}$ reciprocal ohms. The figures have been calculated to the basis of 1,000. The results show that the equations

TABLE VII.

Rate of Hydrolysis.

Substrate = 2.0 per cent egg albumin solution, pH 2.0, = 10.0 units of pep-
tone per cc. when completely hydrolyzed (from Table III).

Pepsin = 0.02 per cent = 0.2 units per cc.

Temperature 38°C. $A = 1,000$.

Time. Hrs. $\times 10^2$	Increase in resistance. = X	Values of $K = \frac{X}{\sqrt{ET}}$	Values of $K = \frac{A \ln \frac{A}{A-X} - X}{ET}$
1	1	1	0.06
7	43	16	0.14
11	62	18.7	0.23
18	118	28	0.42
22	143	30	0.51
26	183	36	0.73
31	212	38	0.85
36	240	40	0.94
41	260	40	1.00
51	282	39.5	1.00
61	335	43	1.17
73	360	42	1.17
97	415	42	1.17
125	454	40	1.18
260	582	36	1.12
362	652	34	1.10
462	690	32	1.0
562	740	31	1.1
3,600	1,000		

fit the experimental results almost exactly as was predicted from the derivation. The decline in the value of the constant near the end of the reaction of equation (6) is due to the fact that the changes in conductivity of the solution do not accurately represent the digestion at the end of the experiment.¹⁹ It will be shown later that, when

¹⁹ It has been shown in another paper in this *Journal* (Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 475) that the destruction of pepsin under the conditions of this experiment is so slight as to be negligible. The decrease in the rate of reaction cannot be ascribed to this cause.

the changes in digestion are followed by means of the increase in amino nitrogen, which probably accurately follows the digestion, equation (6) gives a constant value for K .

Equation (6) is identical with that derived by Arrhenius¹⁶ from the action of ammonia on a great excess of ethyl acetate, and applied by him to peptic digestion. Arrhenius, however, considered A as representing the concentration of ammonia (which would correspond to the concentration of pepsin in these experiments). In other words

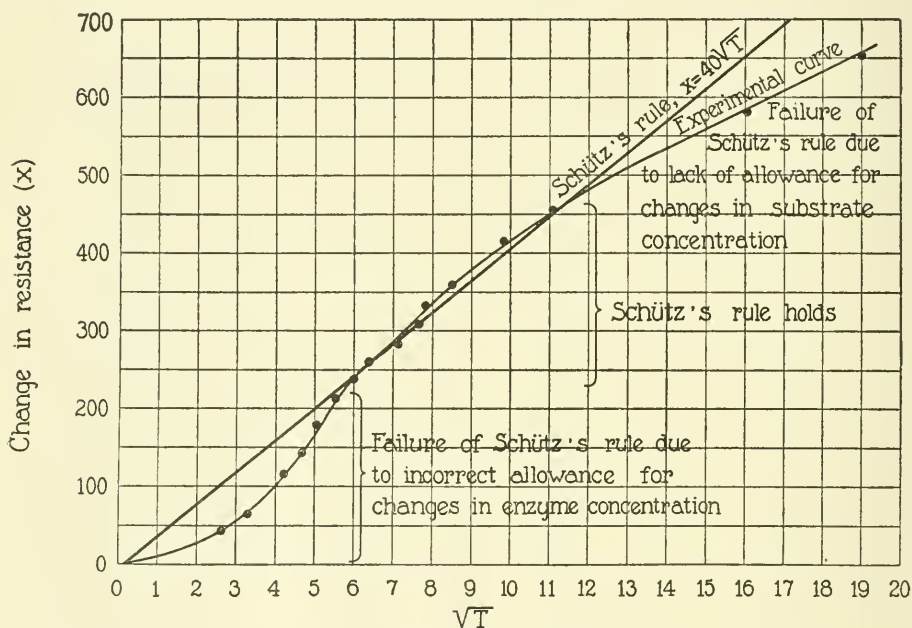


FIG. 5. Curves showing rate of digestion of egg albumin (*cf.* Table VII).

the entire term $\frac{(A-x)}{x}$ in Arrhenius' equation represents the ammonia (or enzyme) concentration while the substrate concentration is considered to remain constant. It is clear from the derivation of the equation presented in this paper, however, that the term $\frac{E}{x}$ represents the enzyme concentration, while $A-x$ represents the substrate concentration. The equation as applied to the hydrolysis of ethyl

acetate by a small amount of ammonia or to the hydrolysis of protein by pepsin is therefore identical in form but differs as to the significance of the term $A - x$.

In all the foregoing experiments the rate of digestion has been followed by means of changes in the conductivity of the solution. Since this value does not accurately represent the course of digestion, the objection might be raised that the agreement between observed and calculated values is due to compensating errors in the derivation of the equation and in the deviation of the conductivity changes from the actual progress of digestion. This could not be the case in the tests of the equation in the differential form since in this case the results are comparative and any deviation of the conductivity changes from the true rate would cancel out. It is possible, however, that the agreement of the equation in the integral form might be due to some such compensation of errors. In order to show that this is not the case a series of experiments was made in which the course of digestion was followed by means of the increase in amino nitrogen. This value was determined by Van Slyke's²⁰ method as already described,²¹ and, as far as is known, accurately represents the progress of digestion. In these experiments the quantity of egg albumin was kept constant (0.5 per cent egg albumin) and the concentration of pepsin varied. The results are summarized in Table VIII. The figures given under X are the increase in amino nitrogen in cc. per 660 cc. of solution. They are the average of three determinations and have an experimental error of about 10 per cent. This is sufficient to account for the variations in the constant of equation (6). The errors in x are greatly magnified in this constant as it depends on the difference between two experimental values.²² In every case sufficient time had elapsed before the first observation so that x at the time this determination was made was already large compared to the concentration of pepsin. The change in value of the constants for the first minutes of the re-

²⁰ Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

²¹ Northrop, J. H., *J. Gen. Physiol.*, 1918-19, i, 607.

²² The errors in x are reduced in the constant of Schütz's rule. The variations in this constant are therefore outside the limits of experimental error while those of Arrhenius' constant are within the limits of experimental error.

TABLE VIII.

Comparison of Schütz's Rule and Arrhenius' Equation. Rate of Hydrolysis.

Substrate = 0.5 per cent egg albumin solution, pH 2.0.

 X = relative increase in amino nitrogen per cc. of solution. E = relative pepsin concentration.

Time.	X	E	$K = \frac{X}{\sqrt{E T}}$	$K = \frac{\left[A \ln \frac{A}{A - X} - X \right]}{E T}$
<i>min.</i>				
30	380	32	12.0	0.10
75	500	32	10.0	0.08
135	640	32	10.0	0.08
255	700	32	8.5	0.05
495	806	32	6.6	0.05
840	860	32	5.0	0.04
1,740	975	32	4.0	0.05
4,200	[1,000]	32		
30	224	16	10.0	0.06
75	382	16	12.0	0.07
135	528	16	11.0	0.10
255	608	16	10.0	0.07
495	620	16	7.0	0.04
840	700	16	6.0	0.04
1,740	860	16	5.0	0.04
4,200	[1,000]	16		
30	152	8	10.0	0.06
75	277	8	11.0	0.08
135	390	8	12.0	0.10
255	516	8	11.0	0.10
495	536	8	12.0	0.06
840	656	8	8.0	0.05
1,740	742	8	6.5	0.06
4,200	930	8	2.3	0.07
30	100	4	13.0	0.045
75	174	4	10.0	0.055
135	225	4	11.0	0.055
255	363	4	11.0	0.08
495	415	4	9.4	0.06
840	490	4	8.5	0.055
1,740	636	4	7.6	0.055
4,200	770	4	6.0	0.045

TABLE VIII—*Concluded.*

Time.	X	E	$K = \frac{X}{\sqrt{ET}}$	$K = \frac{\left[A \ln \frac{A}{A-X} - X \right]}{ET}$
<i>min.</i>				
30	90	2	11.5	0.07
75	139	2	11.5	0.07
135	180	2	11.0	0.07
255	265	2	12.0	0.06
495	305	2	10.0	0.06
840	385	2	9.5	0.06
1,740	500	2	8.5	0.06
4,200	630	2	7.0	0.05
30	80	1	14.5	0.10
75	93	1	11.0	0.06
135	112	1	10.0	0.05
255	165	1	10.0	0.06
495	180	1	8.0	0.03
840	230	1	8.0	0.04
1,740	350	1	8.5	0.05
4,200	420	1	6.5	0.03

action, noticed in Table VII, is therefore lacking here. The results show that the equation gives a fairly satisfactory constant when it is considered that the experimental observations are very difficult and that the experiments represent changes in the value of E , x , and T of many hundred per cent. Individual experiments were made which gave much more constant values for K . The present series is given preference, however, since it shows that the equation takes into consideration changes in the enzyme concentration. It is obvious, however, that this equation is merely an approximation formula which will hold only under certain limited conditions and is but little more general than Schütz's rule. The derivation given offers a rational interpretation of both expressions. It may be pointed out also that equation (6) contains only one arbitrary constant K and can therefore hardly be considered as empirical.

DISCUSSION.

It has been shown in the preceding paragraphs that the divergence of the kinetics of pepsin action, from the results predicted from the

law of mass action, may be quantitatively explained by the assumption that the enzyme in solution is in equilibrium with the products of digestion of the protein, or some other substance, and that this equilibrium obeys the ordinary laws of mass action. The results of Peckelharing and Ringer²³ may be taken as experimental proof that the enzyme is so combined. These authors found that very pure solutions of pepsin showed no isoelectric point when tested between two oppositely charged electrodes; but that the addition of peptone caused the pepsin to change the direction of migration at a pH of about 3.0; which corresponds approximately to the isoelectric point of these added substances. It is difficult to explain this experiment otherwise than to conclude that the pepsin combines with the peptone and is carried with it to the electrode. If some of the pepsin combines with peptone, therefore, and so becomes inactive the rate of digestion will evidently not be directly proportional to the total concentration of pepsin but to some other function of the total concentration as defined by the mass action equilibrium. This is exactly analogous to the relation between the hydrogen ion concentration and the total acid concentration. In this case it is only the hydrogen ion which is active in hydrolysis and the activity of the solution is therefore not directly proportional to the total acid concentration. (In the case of acid it is known that the dissociation is electrolytic; *i.e.*, the dissociated parts of the molecule are electrically charged. Whether this is true or not in the case of the pepsin cannot be stated as yet.) The rate of reaction then becomes directly proportional to the active (free) enzyme concentration as demanded by the law of mass action; and the apparent divergence from this law is due to the fact that the total enzyme concentration and the active enzyme concentration are not always directly proportional; just as the total acid concentration and the active acid concentration are not always directly proportional. If this hypothesis is correct, it seems probable that the enzyme does not combine with the substrate for an appreciable length of time, but that the contact of enzyme and substrate molecule results in immediate decomposition of the latter into its products of digestion. There is no doubt that the enzyme actually does combine with the substrate when the latter is not in solution.⁶ It is quite possible, however, that this is a case of solution of the en-

²³ Peckelharing, C. A., and Ringer, W. E., *Z. physiol. Chem.*, 1911, lxxv, 282.

zyme in the solid phase and that the kinetics of the reaction are the same there as in the liquid phase. There is some experimental evidence in favor of this point of view. It was found that the rate of digestion of edestin was the same when in solution and when suspended in the enzyme solution.²¹ Dauwe²⁴ has shown that pepsin can diffuse through a membrane of solid protein.

The hypothesis enables us to set an upper limit for the purity of an enzyme preparation. It was found, for instance, in Experiment 1, that the enzyme solution used contained about twenty-seven (arbitrary) units of pepsin and about thirty units of peptone. Assuming that the combining weights of the substances are approximately the same it is obvious that the original preparation could not have been more than 50 per cent pure pepsin. It is, however, quite possible that the enzyme may be combined with some substance and still retain its activity (as found for invertase combined with charcoal by Nelson and Griffin²⁵) or that impurities are present which do not combine with the enzyme at all. It is not possible therefore to assume that the active pepsin consists solely of pepsin molecules. For similar reasons it is not possible to draw any definite conclusions from the results of Experiment 2 in which it was found that a 1 per cent egg albumin solution after complete digestion contained about two arbitrary units of peptone while a 1 per cent pepsin solution contained about ten units of pepsin.

It is well known that the kinetics of enzyme reactions differ in another respect from the general laws of chemical reactions in that the rate of reaction in high concentration of substrate does not vary directly with the total substrate concentration. This phenomenon is very similar to the one discussed in the present paper and it would seem that the same explanation applies to both cases; *i.e.*, that the active substrate concentration is not directly proportional to the total substrate concentration.

It may be pointed out that, according to the above mechanism of the reaction, pepsin cannot be considered a catalyst in the sense of the classical definition since it combines with some, at least, of the products of reaction and so enters directly into the equation. Since

²⁴ Dauwe, F., *Beitr. Chem. Physiol. u. Path.*, 1905, vi, 426.

²⁵ Nelson, J. M., and Griffin, E. G., *J. Am. Chem. Soc.*, 1916, xxxviii, 1109.

the enzyme combines with one (at least) of the products of reaction its presence must necessarily affect the equilibrium point. The reaction, therefore, would appear to be a special case of bimolecular reaction in which one of the reacting substances (the enzyme) forms a highly dissociated compound with one of the products. The truth of the matter probably is that so called pure catalytic reactions are merely limiting cases in which the combination of the catalyst is so small as to escape detection (Stieglitz).⁵

SUMMARY.

1. In certain cases the rate of digestion of proteins by pepsin is not proportional to the total concentration of pepsin.

2. It is suggested that this is due to the fact that the enzyme in solution is in equilibrium with another substance (called peptone for convenience) and that the equilibrium is quantitatively expressed by the law of mass action, according to the following equation.

$$\frac{\text{Concentration pepsin} \times \text{concentration peptone}}{\text{Concentration pepsin-peptone}} = K$$

It is assumed that only the uncombined pepsin affects the hydrolysis of the protein.

3. The hypothesis has been put in the form of a differential equation and found to agree quantitatively with the experimental results when the concentration of pepsin, peptone, or both is varied.

4. Pepsin inactivated with alkali enters the equilibrium to the same extent as active pepsin.

5. Under certain conditions (concentration of peptone large with respect to pepsin, and concentration of substrate relatively constant) the relative change in the amount of active pepsin is inversely proportional to the concentration of peptone and the equation simplifies to Schütz's rule.

6. An integral equation is obtained which holds for the entire course of the digestion (except for the first few minutes) with varying enzyme concentration. This equation is identical in form with the one derived by Arrhenius¹⁶ for the action of ammonia on ethyl acetate.

7. It is pointed out that there are many analogies between the action of pepsin on albumin solutions and the action of toxins on an organism.

THE DARK ADAPTATION OF THE HUMAN EYE.

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(Received for publication, February 10, 1920.)

I.

The phenomenon of retinal adaptation is one of the most familiar facts of sensory physiology. Not only is the mere adaptability of the human eye well known, but, since Aubert's (1865) first measurements, there has grown up a body of quantitative data describing the *course* of adaptation (Nagel, 1911). This is especially true of the adaptation of the eye to dim lights.

All the data on dark adaptation show that on entering a dark room after a stay in the outside daylight the eye at once begins to increase in sensitivity. At first this increase appears to be slow; but after 5 minutes the increase is quite rapid, the eye acquiring a sensitivity several hundred times its initial value. After 30 minutes sojourn in the dark the sensitivity still increases, but more slowly than before; and after 45 minutes or an hour the maximum sensitivity is reached. The final sensitivity varies slightly with different people, but in the fully adapted condition the eye is easily 5,000 or 10,000 times more sensitive than it was at the beginning.

In Fig. 1 is given the record of a dark adaptation experiment made by Piper (1903). The results are representative of the numerous published experiments. Piper points out that, although earlier workers (*e.g.* Aubert, 1865) believed the rate of adaptation to be greatest at first, the greatest increase in sensitivity really occurs in the middle of the course of adaptation. The curve of sensitivity according to Piper shows three parts: an initial slow phase, an intermediate rapid one, and a final phase ending in a maximum.

In spite of our familiarity with the phenomena of dark adaptation, and the trustworthiness of the measurements describing them, their

theoretical bearing is practically *nil*. The changes in sensitivity are remarkably constant, not only in a given individual, but in different individuals as well. Age does not change the regularity of the course. The effect of drugs is practically negligible. Even persons possessing deficiencies of color vision present a normal type of dark adaptation. Still the meaning of this uniformly regular change in sensitivity has remained obscure. The course of dark adaptation has not given us a hint of the physicochemical basis of visual reception, though it is apparent that the two phenomena must be fundamentally related to each other.

What are the causes of this failure? In order to answer this question profitably we must consider first the nature of the published data, and second the obstacles in the way of their interpretation inherent in the data. Extensive summaries of the literature of retinal adaptation have been made (Tschermak, 1902; Nagel, 1911). Such is not my purpose. It is rather to analyze the data, and if possible to find some explanation of the pronounced regularity evident in every experiment on the dark adaptation of the human eye.

II.

The pioneer experiments of Aubert (1865), followed by those of Charpentier (1886) and Treitel (1887), demonstrate the qualitative fact that dark adaptation follows a definite course. Due to matters of technique, however, they are not sufficiently accurate to stand on a par with the later experiments of Piper (1903) and Nagel (1911). We shall therefore confine ourselves to the work of the latter investigators. Piper in particular has published the complete results of the retinal adaptation of eighteen people. These detailed data are invaluable in the quantitative treatment of the material.

The experiments consist in finding the intensity of a square area of light which is just barely visible to the eye. Observations are made at regular intervals during the stay in the dark. The subject fixes his eyes on one corner of the square of light, so that most of the light falls on the retina outside the fovea centralis. What one finds is this. At first this minimum intensity is large; as the stay in the dark is prolonged it becomes less and less; and finally it reaches a constant minimum.

Not content with the mere statement of such facts, the investigators beginning with Piper have presented their data in terms of sensitivity or *Empfindlichkeit*. As used in this connection these two words signify some multiple of the reciprocal of the minimum intensity. The actual units of sensitivity vary. Piper uses a million times the reciprocal, whereas Nagel considers *Empfindlichkeit* as the simple reciprocal of the minimum intensity. It is apparent, however, that the two are essentially the same thing. The data shown in Fig. 1 are given in terms of Piper's units of sensitivity.

It is here that we meet the first difficulty. Sensitivity as defined in this way possesses no meaning other than that inherent in the original fact of the minimum intensity. It is true, speaking in a general way, that the irritability of the eye increases as the minimum intensity necessary to stimulate it decreases. But we must not be deceived by so seductive a word as sensitivity, even when it is accompanied by certain figures purporting to represent the number of units of this condition. It is so easy to forget this, and to apply the term as a quantitative estimate of the condition *inside* the eye (*Empfindlichkeit der Netzhaut*) instead of remembering that it applies merely to the condition of the *outside* light. *Empfindlichkeit* includes nothing more than what is implied in the minimum intensity necessary to elicit a visual effect in the eye.

However, even as a statement of the changes in the external light, the use of *Empfindlichkeit* or sensitivity is attended with the danger that it distorts the actual course of retinal adaptation. As a matter of fact the shape of the curve in Fig. 1 and its division into three phases represent neither the properties of the retina nor those of the light. It does represent a certain property of numbers. The whole thing depends on the simple fact that, as a number decreases, its reciprocal increases in a curious way. For example

$$y = x$$

is the equation of a straight line. However

$$y = \frac{1}{x}$$

that is, using the reciprocal of x is not the equation of a straight line at all, but that of an equilateral hyperbola.

From Fig. 1 it might really be supposed that practically no change occurs during the first 5 minutes of dark adaptation. Nothing is farther from actual fact. During this interval the minimum intensity necessary for a visual stimulus,—the visual threshold,—drops to less than half its initial value. In the experiment of Fig. 1 this

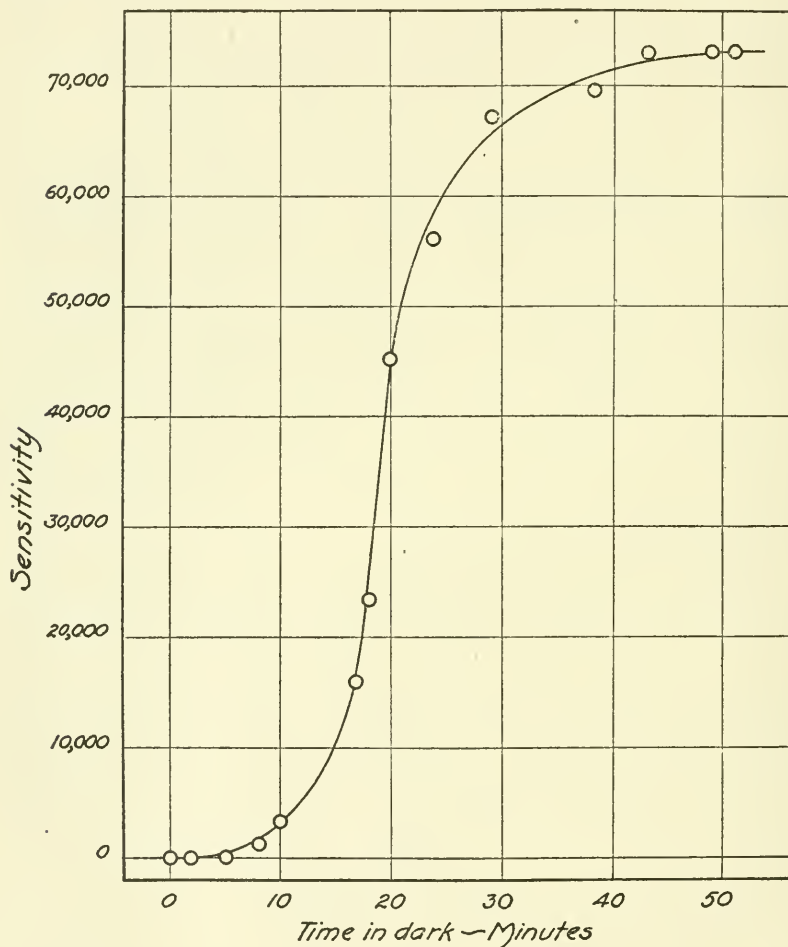


FIG. 1. The course of dark adaptation expressed in terms of the increase in sensitivity. This experiment is typical of the many which have been published. The ordinates are a million times the reciprocal of the minimum intensity; the abscissæ the time in the dark.

decrease amounts to no less than 61 per cent of the initial threshold value. A glance at Fig. 2 and at Table I, presented later in this paper, shows that the minimum intensity,—the data actually obtained,—varies in no such way as indicated by Fig. 1. The data of Fig. 2 are the same as those forming the basis of Fig. 1.

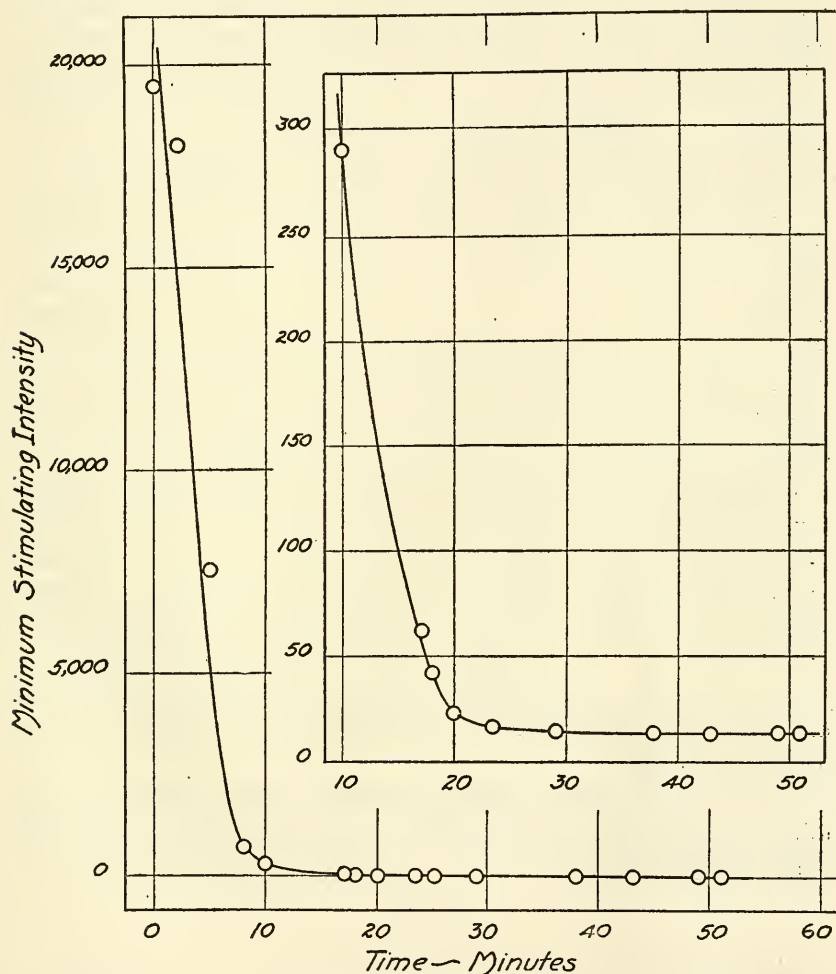


FIG. 2. The course of dark adaptation expressed in terms of the minimum intensity visible to the eye. The data are the same as in Fig. 1. The lower portion of the curve is redrawn in the inset, using a magnified scale of ordinates. The exceedingly great change in the intensity during the first 10 minutes is clearly apparent.

Unfortunately all the investigators following and including Piper have published their results as *Empfindlichkeit*, without giving the minimum intensities as they found them. This has been the most obvious impediment in the way of a proper treatment of their data. The undue emphasis on the distorted results has detracted from the regularities evidenced by the actual data themselves. It is, however, simple in all cases to compute backwards and to find in this way what the original facts are. By taking the reciprocal of the published values of the *Empfindlichkeit* I have calculated the corresponding minimum intensities for most of the published experiments. It is not possible to state what the unit of intensity is in Piper's experiments. I cannot find its definition anywhere in his article. By inference from the work of other investigators I judge it to be about 4×10^{-7} meter candles. The unit of sensitivity in Nagel's adaptometer is definitely stated as the reciprocal of the intensity as measured in meter candles. However, in order to make these data comparable to Piper's, as well as to avoid the use of long decimals, I have multiplied the minimum intensity by 10^7 , thus making the unit of minimum intensity in Nagel's data as 1×10^{-7} meter candles.

The results as we find them now are represented by Fig. 2, which is the same experiment as Fig. 1. It is not possible in a single drawing to show how the intensity varies throughout the test. I have therefore redrawn the lower part of the curve in Fig. 2 using a magnified scale of ordinates to show the changes which take place after the first 10 minutes. It is obvious, as Aubert originally maintained, that the process of adaptation begins immediately, and that the minimum intensity decreases enormously during the first few minutes in the dark.

With the data in their present form we may now proceed to determine what the peculiarities inherent in them are which prevent their ready interpretation.

III.

The experiments on retinal adaptation are a series of determinations of the visual threshold in dim light. Each test is a measurement of the minimum energy for a sensory effect. The interpretation of the findings must then hinge to a large extent on the phenomena that

attend the sensory reception of lights of low intensity. At present we know very little of the basic reactions, chemical and physical, that underlie retinal stimulation. One thing, however, may be stated with considerable confidence. This is that the initial effect of the light on the retina consists of the photochemical alteration of some photosensitive substance. Each test of the visual threshold involves a primary photochemical effect. The data of dark adaptation, if properly treated, should give us information about this photochemical change.

Objectively stated, the facts are that as adaptation proceeds, less and less light energy is necessary to produce the initial photochemical effect required for a visual response. In other words, progressively less and less of the photosensitive substance must be decomposed in order to initiate the subsequent processes concerned in photo-reception.

Granting this qualitative decrease, what are its quantitative implications? We have the minimum intensities from the data. What is the relation between the intensity of the stimulating light and its objective effect in the photochemical decomposition of the sensitive material of the retina? An exact relation undoubtedly exists between the two. Its quantitative expression must be known if the adaptive changes in the retinal mechanism are to be stated objectively. Unfortunately this condition cannot be fulfilled from any experiments on the retina. We have here, then, the first of the inherent difficulties in our knowledge that prevent the proper fundamental interpretation of retinal adaptation.

This, however, is not the only obstacle. Let us tentatively assume that the relation between intensity and photochemical effect is known quantitatively. In this way we will know the exact quantities of photosensitive substance required to be decomposed in order to initiate a minimal retinal effect. This follows from the fact that the photochemical effect E is some function of the intensity I

$$E=f(I)$$

even though the exact nature of the function is not stated.

During adaptation in darkness the amount to be decomposed changes regularly. What determines the magnitude of the quantity

of decomposed material necessary for the initiation of a visual effect at a given moment? This really amounts to a demand for the objective basis of variations in the irritability of the retinal mechanism. We must know why at any given moment a certain number of units of decomposed photosensitive material is necessary for the production of a visual response, before the strikingly regular variations of these quantities can attain any basic significance. Here we meet with the second obstacle in the way of an interpretation, because the question cannot be cleared up in terms of the existing data of retinal physiology. Indeed it is difficult to conceive of experiments on the vertebrate retina so designed as to give an objective answer to these two questions.

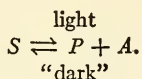
It must be remembered that the important point of the data of retinal adaptation is not merely the fact of adaptation, but the consistently regular sequence in the course of adaptation. Given the means of answering the two questions relating to visual reception, this orderly progress of dark adaptation might be attacked with profit. Lacking them, it is small wonder that the data are meaningless in themselves, and that they have failed to add to a possible hypothesis for the basis of visual reception.

IV.

Although retinal physiology has not been able to surmount the difficulties previously enumerated, there are some experiments recently made with invertebrates that may help in this connection. The work on the light sensibility of *Mya* and *Ciona* (Hecht, *a, b, c, d*) has demonstrated two aspects of the sensory process which are intimately connected with the problem of retinal adaptation. The first of these is concerned with the relation between the intensity of the stimulating light and its photochemical effect in photoreception. The second presents an objective basis for the meaning of variations in irritability.

According to the hypothesis suggested for it, the photosensory mechanism in *Mya* is composed of two processes, one following the other in point of time. The initial process is a photochemical reaction; the subsequent one is an ordinary chemical reaction which is

catalyzed by the products formed in the photochemical reaction. It is the initial photochemical reaction that is of interest in the present context, and with it alone we shall be concerned. Without going into details,—for which the original work must be consulted,—the results are as follows. A photosensitive substance S is decomposed by the action of light into two products of decomposition P and A . This reaction is reversible, and in the absence of light the reverse reaction goes on unopposed by the light reaction. The equation for the complete process is thus



It is apparent that the substances P and A are the precursors as well as the decomposition products of the photosensitive substance S .

The velocity of the light reaction, $S \rightarrow P + A$, is entirely dependent on the intensity of the incident light. The exact relation between the two is a logarithmic one. If E is the photochemical effect, as measured by the amount of P and A formed in unit time, and I the intensity of the light, then

$$E = k \cdot \ln I$$

\ln being the sign of natural logarithms. In *Mya*, k has a value of 1. This quantitative relation is the first of the two aspects of the sensory process to which reference has been made. Its significance lies in its ability to describe the action of light in the objective terms of a physicochemical mechanism, rather than in terms of sensory effects.

The second feature of the photosensory mechanism as postulated for *Mya* and *Ciona* is also concerned with products of the light reaction $S \rightarrow P + A$. This second principle states that the degree of irritability of the sense organ depends entirely on the concentration of the precursor decomposition products present in the sensory mechanism. To be more precise: before it can cause a sensory effect, the incident light must produce such an amount of freshly decomposed precursors P and A that a definite ratio is attained between the freshly formed and the residual precursors present in the sense organ.

The importance of this concept cannot be overemphasized, because it lies at the foundation of all the work with *Mya* and *Ciona*. Together with the logarithmic relation previously explained, it enables one to visualize the initial chemical events necessary for the production of a sensory effect in these animals.

V.

If the initial photochemical reaction in retinal sensitivity has something in common with photoreception in *Mya*, then the application of the findings with *Mya* to the data of visual adaptation should yield results of theoretical bearing. The data give the intensities of the light necessary for a visual effect. The photochemical action of this light should therefore be represented by the logarithm of its intensity. These logarithmic values will then give the actual number of units of photosensitive substance decomposed by the light, because

$$E = k \cdot \log I.$$

We do not know what the value of the constant k is here. But we can always make it equal to 1 by changing the units in which the photochemical effect is measured. Such a change makes no difference in our conclusions, because any unit is a purely arbitrary thing.

The data treated in this way are given in Figs. 3 to 8. These figures are different experiments taken from the work of Piper (Figs. 3 to 7) and of Nagel (Fig. 8). An example of the procedure necessary in calculating the data from the published experiments is given in Table I. The first two columns are from the published results. The last two columns are the computed values of the original intensities and their logarithms. The data of Table I are given graphically in Fig. 3.

In the presentation of these experiments I have exercised a certain amount of selection in the following way. Some of Piper's experiments are vitiated by the fact that the measurements are made too frequently. For example, in one case nine determinations are recorded in 11 minutes, some having been made only $\frac{1}{2}$ minute apart. Considering that even a small flash of light retards dark adaptation, it is hard to understand why such an error should have been com-

TABLE I.

Data of an Experiment on Dark Adaptation of the Eye.

Time.	Sensitivity.	Intensity.	Logarithm of intensity.
<i>min.</i>			
0	38.7	25,840.0	4.41
2	99.7	10,030.0	4.00
5	398.7	2,506.0	3.40
8.5	1,065.0	934.6	2.97
12	3,420.0	292.4	2.47
20	16,870.0	59.9	1.78
31	30,780.0	32.5	1.51
39.5	45,270.0	22.1	1.34
45	54,080.0	18.5	1.27
72	54,080.0	18.5	1.27

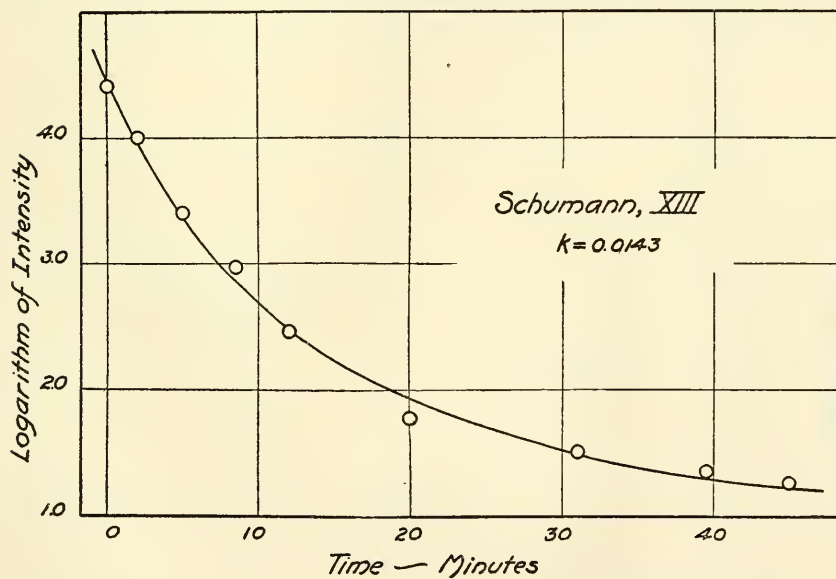


FIG. 3. Dark adaptation of the eyes of Dr. Schumann, as determined by Piper. Schumann has an anomalous trichromatic vision.

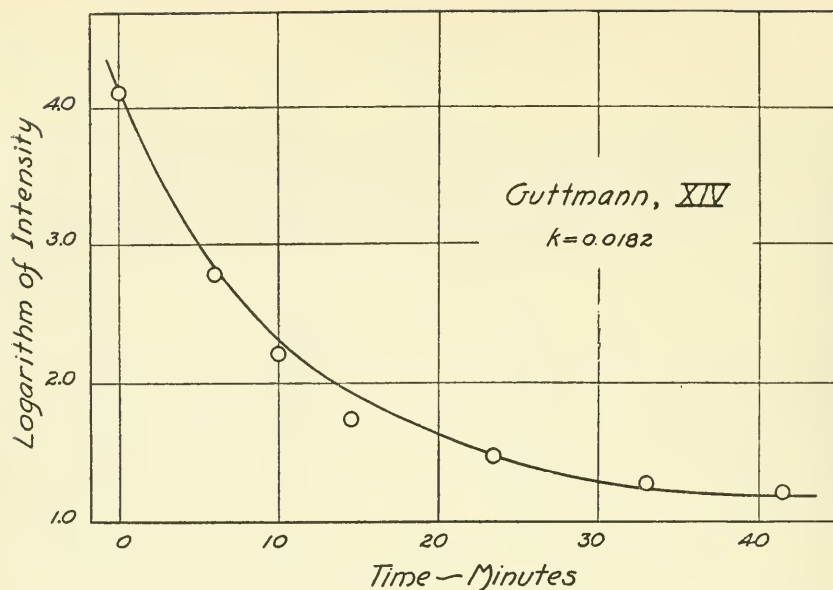


FIG. 4. Dark adaptation of the eyes of Dr. Guttman, determined by Piper. Guttman is also an anomalous Trichromat.

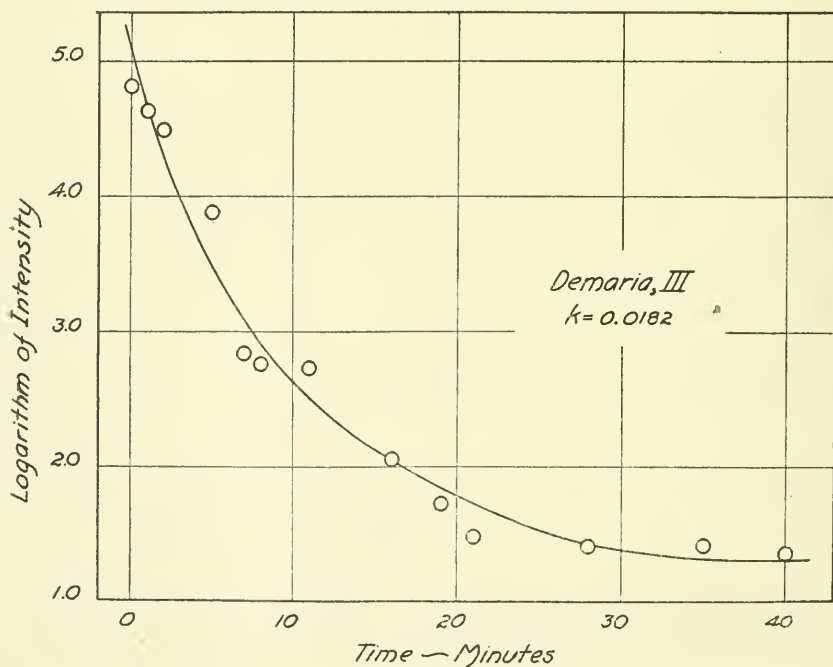


FIG. 5. Dark adaptation experiment with Dr. Demaria as subject. Normal trichromatic vision. Piper's data.

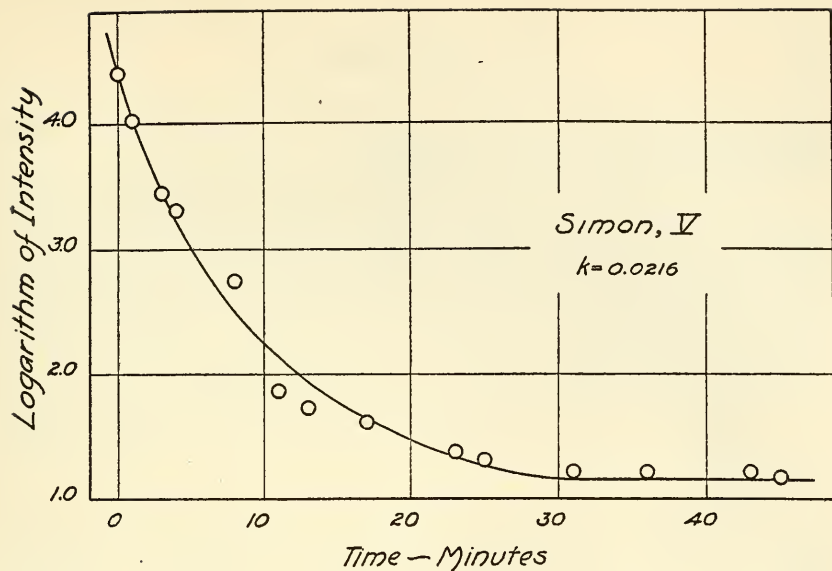


FIG. 6. Dark adaptation of Dr. Simon's eyes, determined by Piper. Simon's vision is that of a normal Trichromat.

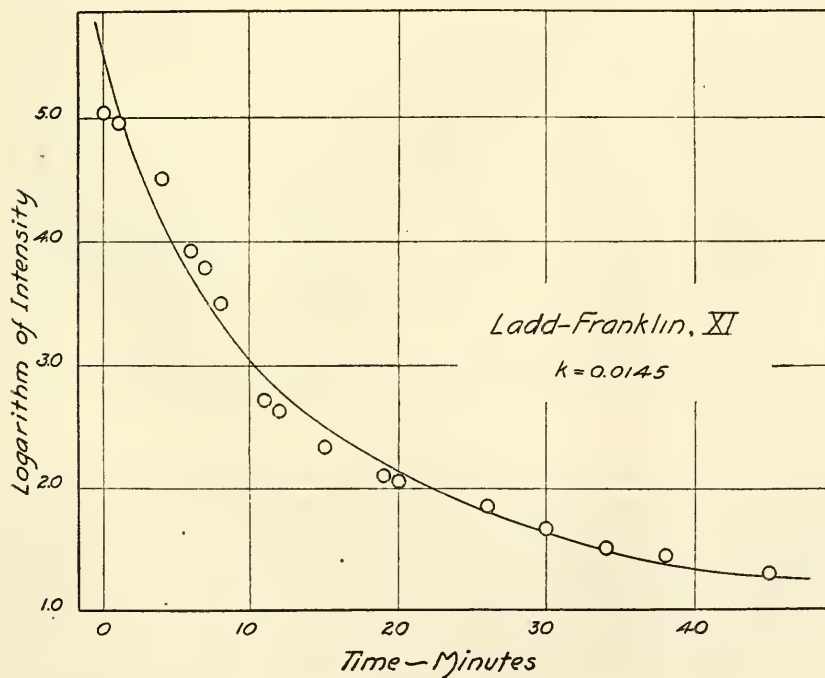


FIG. 7. Adaptation experiment with Mrs. Ladd-Franklin as subject. Normal trichromatic vision. Piper's data.

mitted. I have therefore chosen those experiments which are practically free from this error of technique.

The ordinates in Figs. 3 to 8 are given as the logarithms of the intensity in order to present the data. They really must be interpreted, however, as the number of units of photochemical decomposition products freshly formed by the light, because of the logarithmic relation between the two. As such, what do they tell us about the process of adaptation?

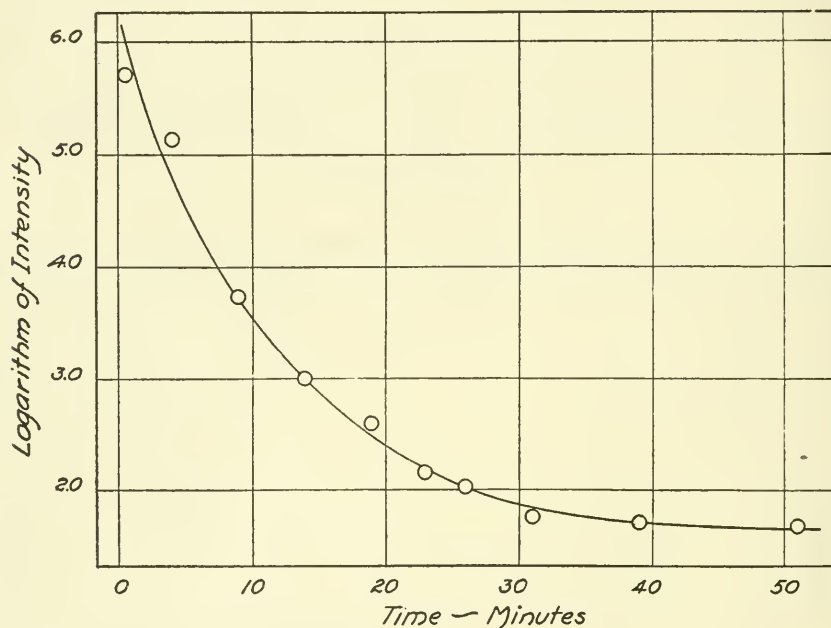


FIG. 8. Dark adaptation experiment recorded by Nagel. The units of intensity are different from that of the previous figures. The results, however are the same.

We learned from *Mya* that the amount of freshly formed decomposition products must be a definite proportion of the amount of these products already present in the sense organ before they can initiate a sensory effect. If we know the required amount of freshly formed products, we therefore know the amount of the residual products, because the ratio between the two is constant. The necessary quantities of freshly decomposed photosensitive material are

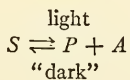
thus a direct measure of the amount of decomposition products at that moment in the retina. The points in Figs. 3 to 8 then give the concentration of the residual decomposition products.

The data now assume a dynamic aspect. Each figure represents the changes in the concentration of the residual decomposition products during adaptation. It is apparent that the concentration steadily decreases. The decrease is regular and follows the course of a chemical reaction. In order to show this the curves in all the figures are the isotherms of a bimolecular reaction. The equation for the curves is

$$k = \frac{1}{a \cdot t} \cdot \frac{x}{a - x}$$

the values of k for each experiment being given in the respective figures. It must be emphasized that each of the points in these figures represents only a *single* measurement. The agreement between the individual experimental values and the theoretically calculated curves is truly striking.

The fact that the curves are reactions of the second order is significant. *Two* products of decomposition are diminishing in concentration in a manner which shows that they are combining to form a chemical compound. These two products are originally the results of the decomposition of a photosensitive substance. A simple explanation is that the compound formed by their chemical union is identical with the photosensitive substance from which they were formed. Such an interrelation is quite common in photochemical reactions. Using S to denote the photosensitive substance, P the principal product of decomposition, and A its accessory, we may write



as the equation of the photochemical reaction. This reaction forms the initial step in the visual reception of dim light by the human retina. The substances P and A are thus the precursors as well as the decomposition products of the photosensitive substance S , the three forming the components of a completely reversible reaction (*cf.* Hecht, 1918-19, *a*, p. 161).

VI.

In terms of such a reversible photochemical process the phenomena of dark adaptation are fundamentally simple. During the stay in the light, a large amount of photosensitive substance is decomposed according to the reaction $S \rightarrow P + A$. The rate of this decomposition will depend entirely on the intensity of the light. The reverse, "dark" reaction $P + A \rightarrow S$, being an ordinary chemical reaction, will proceed whenever any products of decomposition are formed. According to the mass law the rate of synthesis of S from P and A will depend on the concentration of the latter two substances. Between the two opposing reactions a stationary state will be reached (*cf.* Weigert, 1911, p. 15, for the difference between a stationary state and a condition of true equilibrium). This stationary state will represent a definite concentration of the three components, and will depend entirely on the light intensity.

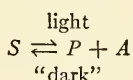
Removal into the dark at once causes the light reaction, $S \rightarrow P + A$, to stop, leaving the "dark" reaction, $P + A \rightarrow S$, to go on unopposed. The continuous action of this "dark" reaction then determines the course of dark adaptation. In order to measure the irritability during dark adaptation a visual test is used. The necessary light decomposes S into fresh P and A . The ratio between fresh and residual P and A is constant. Therefore as the residual products of decomposition disappear, less and less fresh P and A are required to initiate a visual effect. In other words, the retina becomes more and more irritable, as we already know.

The fact that the eye may become adapted to any intensity of light finds its explanation in the stationary state of the opposing chemical reactions. This is entirely a function of the intensity of the light, provided the temperature remains constant. At high intensities the concentration of P and A during the stationary state will be much greater than at lower intensities. This means that the light required to produce a minimum visual effect will vary similarly. Thus the sensory threshold will be higher at higher intensities than at lower, which again is a truism of retinal physiology (*cf.* Hecht, 1918-19, *b*, p. 553).

It is not my purpose to show how many of the properties of visual reception may be explained in terms of the reversible photochemical reaction postulated for the initial effect in photosensory reception. Outside of dark adaptation, this is possible only in a qualitative way, because much of the needed data does not exist in quantitative form. It is clear, however, that the consequences of the reversible reaction may be calculated and predicted, and experiments devised to test the possibilities. Such quantitative results will be forthcoming in the future, and their analysis will be reserved for that time.

One caution must be mentioned. The provisions of the Duplicity Theory make a clear distinction between vision in dim light and vision in bright light (Nagel, 1911). Dark adaptation is essentially a phenomenon of dim vision. Therefore all our conclusions must be limited to the mechanism of vision at low intensities only. The properties of photoreception at high intensities, involving as it does color vision, cannot be considered at the present time. It will be remembered that defects in color vision cannot be correlated with any changes in the course or the quantity of dark adaptation (Piper, 1903, p. 191). Indeed the results given graphically in the present paper represent not only normal Trichromats, but anomalous Trichromats as well.

The fact that our analysis applies only to vision in dim light is of considerable advantage in one respect. This concerns the final meaning of the terms in the equation



for the photochemical reaction of photoreception. With vision in dim light there has been associated the existence of visual purple. The evidence for the connection of visual purple with vision is quite striking (Trendelenburg, 1911; Henri and des Bancelles, 1911). The most significant facts in this respect are those concerned with the threshold of sensitivity, the photochemical action of light on visual purple, and the absorption of energy by visual purple. It is known that spectral light falling on the retina, when so diminished in intensity as to be barely visible to the dark-adapted eye, produces a

sensation of light without color. The minimum stimulating energy at different wave lengths has been determined. Similarly the minimum energy necessary to produce a bleaching effect on visual purple has been measured. And finally the light absorbed by the pigment at different wave lengths has also been described. When put into graphic form, the curves of these three measurements all follow the same course (Henri and des Bancel, 1911). This is a powerful argument for the participation of visual purple in the process of photosensory reception.

The reversible character of the chemical behavior of visual purple is well known (Kühne, 1879). The bleached pigment in the retina, and even *in vitro* under certain conditions, regenerates its color when placed in the dark. It may therefore be quite possible that the photosensitive substance *S* which our analysis requires is really visual purple. If this is true, it follows that visual purple when bleached by light breaks down into two substances. An investigation of the dynamics of the regeneration of visual purple will furnish deciding evidence for the identification of visual purple with the hypothetical substance *S*. It is not necessary to suppose that the decomposition and the synthesis represent elaborate changes. A process of reduction, or oxidation, or perhaps of hydrolysis may accomplish all that is required in the way of chemical changes. In fact, considering the extremely small quantities of energy necessary to produce a visual effect, it must follow that the initial photochemical transformation is not only delicate but very simple as well.

The significant point of all this is that the analysis of dark adaptation with which this paper has been concerned is consistent with what we know of the changes in the eye. If further experiments will show the identity of visual purple with the hypothetical photosensitive substance, it will be a distinct advance in our knowledge of the basis of visual reception.

SUMMARY.

During the dark adaptation of the human eye, its visual threshold decreases to a small fraction of its original value in the light. An analysis of the quantitative data describing this adaptation shows that it follows the course of a bimolecular chemical reaction. On the

basis of these findings it is suggested that visual reception in dim light is conditioned by a reversible photochemical reaction involving a photosensitive substance and its two products of decomposition. Accordingly, dark adaptation depends on the course of the "dark" reaction during which the two products of decomposition reunite to synthesize the original photosensitive substance.

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THE SIGNIFICANCE OF THE CAMBIUM IN THE STUDY OF CERTAIN PHYSIOLOGICAL PROBLEMS.

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PLATE 1.

(Received for publication, January 20, 1920.)

INTRODUCTION.

During the last few years, I have had occasion to devote considerable attention to the study of the cambium of the higher plants, in endeavoring to determine to what extent variations in the structure of timber affect certain of its physical and mechanical properties. In conducting these investigations, I have been impressed by the fact that the cambium is an unusually favorable medium for the study of certain theoretical problems, particularly the working sphere of the nucleus, the much discussed nucleocytoplasmic-relation, and the dynamics of karyokinesis and cytokinesis. It seems advisable, accordingly, to call attention to certain phenomena¹ in the cytology of this meristematic tissue which may be of general interest to physiologists as well as to cytologists.

Description of the Material.

As is well known, the stems and roots of gymnosperms and dicotyledons increase in diameter through the activity of a jacketing layer of undifferentiated tissue which forms xylem internally and phloem externally. In the case of most gymnosperms and arborescent and fruticose dicotyledons, the initials of this lateral meristem or cambium continue to divide throughout the life of the plant, and their more or less highly differentiated derivative cells constitute the bulk

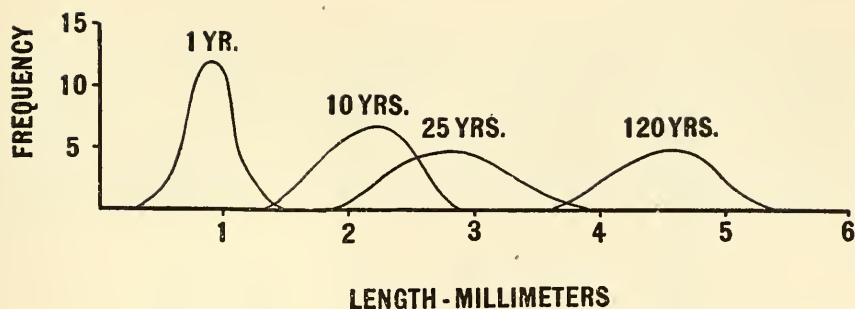
¹ These phenomena will be described and discussed in detail in a series of papers to be published in the *American Journal of Botany*.

of the tissue of an adult individual. The cambial initials are of two distinct shapes and sizes: (1) numerous, large, much elongated (parallel to the long axis of the stem or root) elements; and (2) scattered aggregations of small, more or less isodiametric cells which divide to form the horizontal sheets of radially disposed parenchyma, the so called medullary rays. The principal divisions in both types of initials are periclinal or parallel to tangents to the circumference of the stem or root. In other words, the large cells divide in a tangential longitudinal plane, which is a division plane of *maximal* area, whereas the ray initials form partitions that commonly are surfaces of *minimal* area.

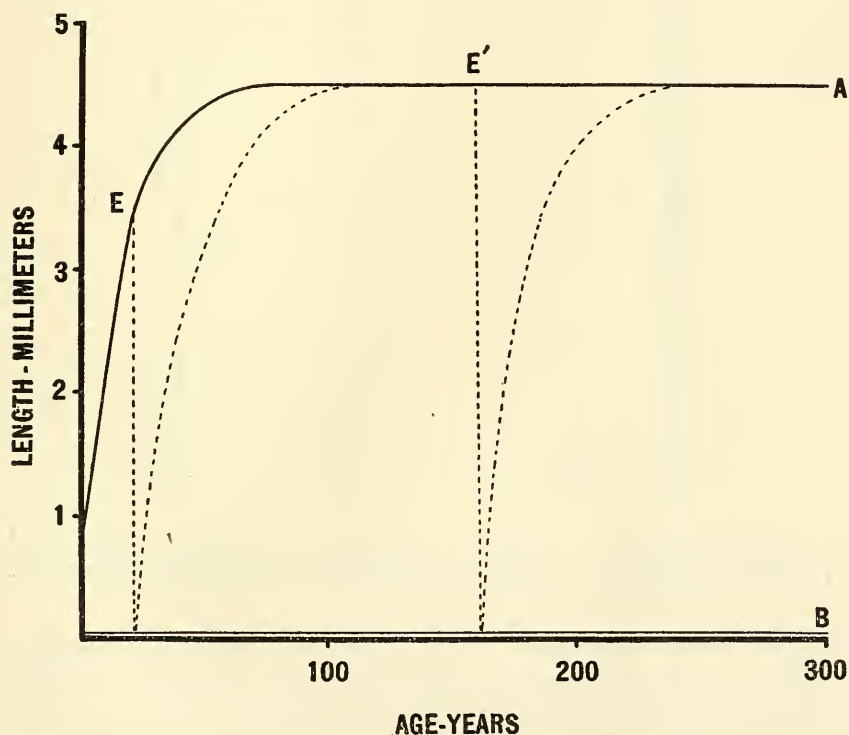
The tangential diameter of the cambial initials increases to a certain extent during the earlier stages of the enlargement of stems and roots, but falls far short of being sufficient to compensate for the rapid increase in the periphery of the cambium. Nägeli inferred from this that the elongated initials must divide periodically in a radial longitudinal plane. He even elaborated formulas for computing the frequency of such divisions during a given increase in the radius of a stem. However, as is not infrequently the case with *a priori* mathematical deductions concerning complex biological phenomena, Nägeli's generalization is supported by few, if any, of the actual facts. Although the hypothetical radial longitudinal divisions are described and figured in many botanical text-books, I have been unable to find them in any of the gymnosperms and less highly specialized dicotyledons that I have studied. The cells slowly elongate, sliding by one another, until they have reached a certain size. They then divide by means of a more or less oblique transverse partition into two short halves, which in turn elongate and divide.² Thus, the increase in the periphery of the cambium is due primarily not to radial longitudinal divisions of the large initials accompanied by lateral enlargement of the products of such divisions, but to the formation of transverse partitions, followed by elongation or longitudinal "sliding growth."³

² It should be kept clearly in mind, in this connection, that during the process of elongation, between successive transverse divisions, the cells continue to divide in the tangential longitudinal plane.

³ This phenomenon of sliding growth is of considerable practical significance in the study of the properties and utilization of timber.

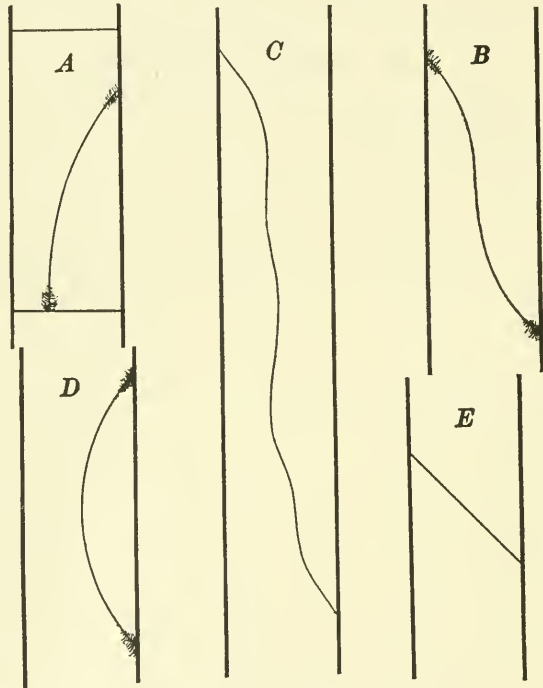


TEXT-FIG. 1. Frequency curves showing variation in the size of cambial initials in stems of different ages.



TEXT-FIG. 2. A, curve showing variations in the average size of large cambial initials with increasing age of a stem. B, size level of cells of the general order of magnitude of the cells of the embryo and growing points. E and E', curves showing effects of experimentally induced changes in cell size.

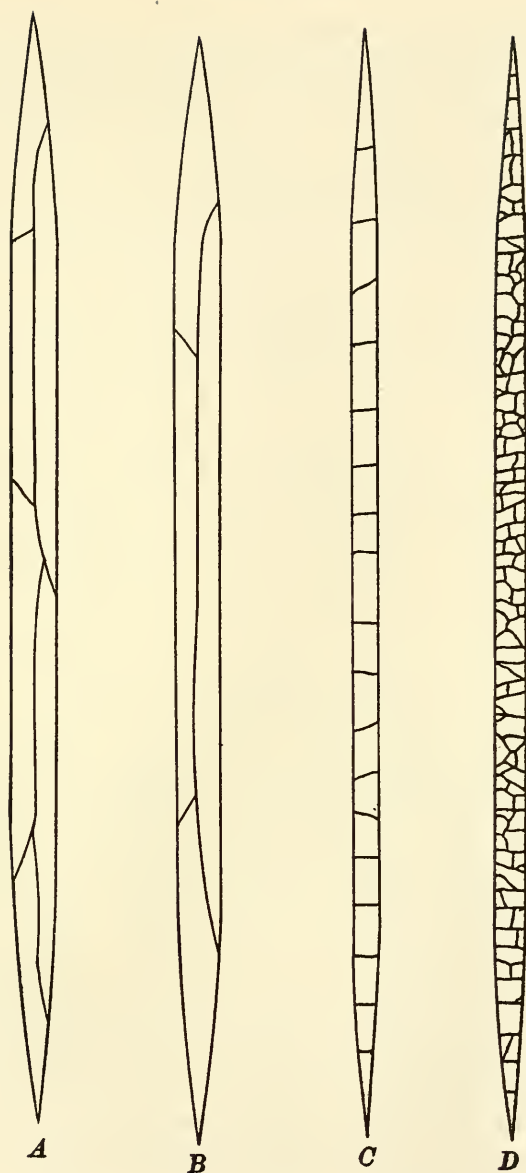
Owing to the fact that the initials do not divide and elongate in unison, there is usually a very considerable variability in the length, and *pari passu* in the volume, of adjacent elements. As shown in Text-fig. 1, the average, maximum and minimum lengths of the cells are considerably less in very young, slender, than in old, robust stems and roots; but the values do not continue to increase throughout the



TEXT-FIG. 3. Acute intersections of division membranes. A, from initial dividing to form callus. B, C, and E, from initials dividing pseudotransversely. D, from initial dividing unequally to form new ray initials.

entire life of an individual. On the contrary, the curve of average length at successive ages is of the general type illustrated in Text-fig. 2 (A). There is a rapid increase in length for a period of years until a certain size is attained, which then remains constant during succeeding growth.⁴ A similar, though much less striking, increase

⁴ The curve for any particular level or radius of a tree usually fluctuates about this norm, owing to the effects of various environmental factors.



TEXT-FIG. 4. A and B, acute intersections of division membranes in the daughter cells of the cambium of certain dicotyledons. C and D, angles of intersection in initials which are dividing to form callus.

commonly occurs in the tangential diameter of the cells; but in the gymnosperms the length of the initials is so great, in proportion to their breadth, that the volume curve closely parallels that for the longitudinal dimension. Under normal conditions the volume of the more or less isodiametric ray initials is very much less than that of even the smallest elongated initials (Figs. 1 and 2) and is of the same general order of magnitude as that of the undifferentiated cells of the embryo and terminal meristems or growing points.

To maintain a proper ratio between the two types of initials, in a layer whose periphery is continually increasing in area, new aggregations of ray initials are periodically carved out of the large initials by appropriate divisions of these elements (Text-fig. 3, D). Furthermore, under abnormal environmental conditions, *e.g.* injuries, all the elongated initials in a given area may be induced to divide into small, more or less isodiametric cells (Text-fig. 4, C and D). During the subsequent growth of this region of the meristem, certain of these small cells elongate and finally regenerate initials of normal dimensions, as shown by dotted lines in Text-fig. 2, E and E'.

The Problem of the Working Sphere of the Nucleus and the Maximum Size of Undifferentiated Plant Cells.

Sachs and Strasburger almost simultaneously called attention to the fact that undifferentiated, actively dividing and growing cells of plants, such as occur in embryonic and meristematic tissue, are relatively minute, and concluded that this was undoubtedly due to the fact that the working sphere of the nucleus is very restricted. Strasburger found that, in the case of "embryonic" cells of the growing points of various plants, the ratio between the average diameters of the nuclei and of the cells is as 0.003–0.016 mm.: 0.005–0.024 mm., or as 2:3; and Sachs pointed out that, although plants vary enormously in their linear dimensions (0.001 to 100,000 mm.), there is not a proportional variation (0.001 to 0.05 mm.) in the size of their constituent cells. Both investigators emphasized the fact that, even in highly differentiated tissues, unusually large or much elongated protoplasts⁵ tend to be multinucleate.

⁵Such as have been critically studied by Schmitz, Treub, Kallen, Johow, Haberlandt, Pirota, Buscalioni, and many others.

In view of these and similar investigations, it might have been expected *a priori* that the large, elongated, undifferentiated cells of the lateral meristem or cambium, which in certain plants attain a length of more than 10,000 micra and a volume of 10,000,000 cubic micra, should contain more than one nucleus each. Such is not the case, however, in any of the somewhat extensive series of gymnosperms and angiosperms that I have studied. Nor do the initials contain abnormally elongated, giant nuclei, such as have been described and figured by Molisch for highly specialized tissue cells of certain monocotyledons. Each initial contains a single nucleus, which is centrally located and retains this position during the processes of growth and cytokinesis.

It is evident, accordingly, that there is a very much greater variability in the size of meristematic cells in plants than was suspected by Sachs or Strasburger, and that in elements of this type the nucleus may extend its "energizing" influence to a distance of several thousand instead of a few micra.⁶

The Relation between Cell Size, Nuclear Size, and Chromosomal Mass.

Strasburger's measurements led him to believe that there is a close correlation between cell size and nuclear size in the meristematic tissue of plants, a conclusion that was strongly supported by the experimental investigations of Gerassimow. The importance of this relation between cell size and nuclear size was further emphasized by Hertwig, and by Boveri, who endeavored to prove that "The size of the larval cells is a function of the quantity of chromatin which they contain, and the volume of the cell is in direct proportion to the number of chromosomes." The subsequent painstaking and detailed investigations of a number of zoologists have indicated that, although in general large cells tend to have larger nuclei than small cells, the nucleocytoplasmic-relation is not invariably a constant and self-regulating ratio. In many animals, it fluctuates within rather wide limits, not only as between different tissues, but even in embryonic

⁶ Sachs considered that the conditions in large animal eggs strengthened rather than weakened his case, since these highly specialized, yolk-containing cells are "inactive" until "energized" by numerous nuclei.

cells during different stages in ontogeny and under different environmental conditions. Conklin has emphasized the fact that in many cases the size of the nucleus is determined by the volume of protoplasm in which it lies, rather than by the number of chromosomes.

On the botanical side, Gates, Gregory, Winkler, and Tupper and Bartlett have shown that a number of races of plants, which have the tetraploid, instead of the diploid, number of chromosomes, are composed of cells larger than those of normal varieties. Winkler's paper is a particularly suggestive one, not only owing to the interesting experimental methods used in his work, but to his general discussion of the relation between cell size and chromosomal number in plants. He reaches the following conclusions, as a result of his own observations and those of a number of other investigators. In embryonic somatic tissue, terminal and lateral meristems, the cells are of nearly uniform size, are roughly isodiametric, and under normal conditions contain the diploid number of chromosomes. Multinucleate protoplasts, nuclear fusions, and changes from the diploid to the tetraploid and polyploid condition are of common occurrence in non-meristematic somatic tissue. In the latter tissue many cells depart widely from the inherited specific cell size of the plant. Such cells tend to be hyperchromatic; much elongated elements containing more than one nucleus each, and other types of large cells an abnormal number (tetraploid or polyploid) of chromosomes. In other words, Winkler considers that there is a very significant correlation between cell size and chromosomal mass, both in the embryonic and non-meristematic somatic tissues of plants.

As I have suggested at the beginning of this paper, the cambium appears to possess certain distinct advantages as a medium for studying various phases of the nucleocytoplasmic-relation. In this tissue adjacent cells vary greatly in shape and size and it is possible not only to compare elongated initials of very different lengths and volumes, but also to contrast them with adjoining ray initials which are of the same general order of magnitude as the cells of the embryo and terminal meristems. By proper experimental methods the long initials may be induced to divide into smaller and smaller units, until more or less isodiametric cells, which resemble the ray initials, are formed. These

may subsequently elongate and regenerate elements of normal size. In regions having cold winters, there is a more or less prolonged resting period, during which the cambial cells are inactive (not undergoing division). This period offers a favorable opportunity for measuring and computing the relative sizes of the initials and their nuclei. Furthermore, during the active growing season, adjacent cells (ray initials and elongated initials of varying sizes) may be found in equivalent stages of karyokinesis and cytokinesis, which facilitates comparisons within the limits of a single section.

TABLE I.
Pinus strobus L.

Type.	Nucleus.				Cell.				Ratio between vol- ume of nucleus and volume of cell.
	Dimensions.			Approximate volume.	Dimensions.			Approximate volume.	
	L	R	T		L	R	T		
	Cambium from 1 yr. old stem.								
Ray initials.....	<i>micra</i> 10.8	<i>micra</i> 8.7	<i>micra</i> 6.5	<i>cu. micra</i> 350	<i>micra</i> 22.9	<i>micra</i> 17.8	<i>micra</i> 13.8	<i>cu. micra</i> 5,000	1:14
Large “	63	3.2	5.8	1,000	870	4.3	16.1	60,000	1:60
	Cambium from 60 yr. old stem.								
Ray initials.....	12.4	12.5	9.9	850	24.8	26.6	17.0	10,000	1:12
Large “	82	5.9	8.9	3,500	4,000	6.2	42.4	1,000,000	1:286

Basis: dimensions of cells and nuclei are averages of 50 measurements.

In 1917, 1918, and 1919, the writer collected numerous specimens of the cambium of *Pinus strobus* L.—from different parts of the stem and root, from trees of different ages and sizes, and from varying environments—at frequent intervals during the resting and growing seasons. As shown in Table I and in Figs. 1 to 6, the larger initials of the common white pine tend to have larger nuclei, but the ratio between cell size and nuclear size fluctuates greatly in the case of the elongated initials. So far as I have been able to determine, however, all the nuclei, regardless of their size, contain approximately the diploid number (twenty-four) of chromosomes. The larger size of the elongated

initials is not associated with a tetraploid or polyploid condition. Nor is it a concomitant of a marked increase in the size of the individual chromosomes, for I have repeatedly found small ray initials in which the chromosomes were fully as long and thick as those of adjoining large, elongated initials. The staining reactions of the various types of initials indicate that the chromatic material is more concentrated in the smaller than in the larger nuclei except during certain stages of karyokinesis; and that the increase in the size of the nuclei is due primarily to an increase in the volume of achromatic substances. Although there is no constant and striking difference in the number and size of the chromosomes in the large and small initials, *the volume of the nucleoli is conspicuously greater* in the larger cells (Figs. 3, 4, 5, and 6). This increase is associated with a *corresponding increase in kinoplasma* during karyokinesis and cytokinesis.

My observations upon *Pinus strobus*, therefore, do not support Strasburger's and Winkler's conclusions in regard to the constancy of "specific" cell sizes and nuclear sizes in the meristems of plants and are in opposition to Winkler's assumption that giant cells are hyperchromatic. In lateral meristems there are relatively great variations in cell size without corresponding changes in the number of chromosomes. In other words, in dealing with the nucleocytoplasmic-relation it is essential to distinguish between (1) those cases in which there is a correlation between cell size, nuclear size, and chromosomal mass, and (2) those in which chromosomal number (chromosomal mass) *is constant and nuclear size and cellular size are variable*.

Karyokinesis and Cytokinesis.

In the gymnosperms, as illustrated by *Pinus strobus*, the polar axis of the division figure usually does not stand at right angles to the long axis of the protoplast, but is placed diagonally across the cell (Fig. 7). This position of the karyokinetic figure is not an artifact, *i.e.* due to displacement of an ordinary spindle, since the whole figure is asymmetrically developed in conformity with its diagonal position. The formation of a cell plate, starting from one of these obliquely placed spindles, is a truly remarkable cytological phenomenon. The spindle becomes greatly extended laterally by the addition of peripheral "fibers," and gradually assumes a more or less curved

form (Figs. 8 and 9). As more peripheral fibers are successively added, the remains of the original central fibers disappear (Fig. 9) from about the cell plate, leaving two separate aggregations of fibers that are connected by the first formed portion of the cell plate (Fig. 10). These aggregations of kinoplasmic fibers, which I have called *kinoplasmasomes*, have a very characteristic form and structure, both in gymnosperms and angiosperms. When the initials are dividing in the usual tangential longitudinal plane, the kinoplasmasomes extend across the cell—at right angles to the longitudinal axis—from one radial wall to the other (Fig. 12) and are located in the center of the protoplast, midway between its tangential surfaces (Fig. 10). In sectional view, they have a somewhat wedge-shaped outline, bluntly convex in front and tapering to a point at the rear along the cell plate (Fig. 10). They move in opposite directions towards the ends of the cell (Figs. 11 and 12). As they move forward the cell plate is extended until it eventually reaches the two ends of the cell, thus dividing the protoplast into two similar halves, each of which contains one of the daughter nuclei (n). The latter remain close together, near the center of the cell, during the process of cell plate formation, and I have been unable to demonstrate any visible connection between the daughter nuclei (n) and the kinoplasmasomes (k) or their constituent fibers. Not infrequently the distance traversed by the kinoplasmasomes, in passing from the vicinity of the daughter nuclei to the ends of the protoplast, may be from 500 to 5,000 micra. The two kinoplasmasomes usually, although not invariably, move forward at *equal rates*, so that at any given stage they appear to be equidistant from the starting point and daughter nuclei. Preliminary investigations indicate that the total time consumed in the process of cell plate formation is considerable, in all probability a matter of many hours rather than minutes.

In the normal, tangential longitudinal divisions of the large initials, the kinoplasmasomes, once they have curved into a position midway between the tangential walls of the cell, move in a straight line towards the ends of the protoplast; thus the division is a plane surface. This is frequently not true, however, in the case of the semitransverse divisions of the initials. The kinoplasmasomes meander more or less in certain cases and curved or undulating surfaces are formed in consequence (Text-fig. 3).

Dynamics of Cytokinesis.

The phenomena of karyokinesis and cytokinesis in the cambium are in direct contradiction to most generalizations concerning cell division. According to Sachs' Law, successive division planes should intersect at right angles; but in the cambium the successive longitudinal partitions are parallel. Hertwig's development of Sachs' Law hypothecates that the axis of the mitotic figures typically lies in the longest axis of the protoplasmic mass, and division therefore tends to cut this axis at a right angle; but in the cambium the axis of the mitotic figure is usually placed either at right angles to the long axis of the cell (angiosperms) or in a diagonal position (conifers, angiosperms). Errera's (Plateau's) Law of Minimal Area is based upon the assumption that the recently formed membranes of cells are semiliquid films which "tend to assume a form which would be assumed, under similar conditions, by a liquid film destitute of weight;" but in the cambium the partitions are commonly division planes of maximal area. Furthermore, according to this law, the division membranes of most plant cells⁷ should intersect the sides of the cells at right angles. De Wildeman, Thompson, and others contend that even the occurrence of oblique divisions in elongated plant cells does not necessarily invalidate the minimal area hypotheses, provided these partitions are sigmoid and intersect the side walls at right angles.

It is significant, accordingly, that in the cambial initials—as also in their daughter cells—of gymnosperms and dicotyledons, many partitions are formed which intersect the older, rigid walls at angles of varying degrees of acuteness (Text-figs. 3 and 4). These acute angles are not due to sliding growth and displacement of protoplasts. The peculiar type of cell plate formation in the cambium facilitates the study of successive stages in the formation of the division membranes and it is evident that the kinoplasmasomes, cell plates, and new cell walls frequently intersect the sides of the cells at very acute angles (Text-fig. 3).

⁷ The older walls of most cells of the higher plants are relatively thick and rigid.

These facts, as those cited by Chambers, raise the question whether in dealing with cytokinesis we actually are concerned with protoplasm in liquid or semiliquid phases.

In any case, this type of cytokinesis, in which the process of cell plate formation is so greatly extended—both as regards space and time—and so clearly dissociated from the usual phenomena of karyokinesis, promises upon further and more critical analysis, to be of some significance in any general discussion concerning the dynamics of cell division.

SUMMARY.

1. The adjacent, undifferentiated, uninucleated cells of the lateral meristem or cambium are of two distinct shapes and sizes: (1) small, more or less isodiametric initials which are of the same general order of magnitude as the cells of the terminal meristem and embryo; and (2) large, elongated initials which in certain cases may attain a length of more than 10,000 micra and a volume of 10,000,000 cubic micra. The large initials may be induced to divide to form small initials, and the latter to regenerate elongated cells of normal dimensions. Thus, the cambium affords an unusually favorable medium for the study of a number of fundamental physiological and cytological problems.

2. A study of the cambium reveals the fact that there is a very much greater variability in the size of meristematic cells in plants than was suspected by Sachs or Strasburger, and that the working sphere of the nucleus is by no means so restricted as assumed by these investigators.

3. Although the larger cambial initials of *Pinus strobus* tend to have larger nuclei, the nucleocytoplasmic-relation varies within wide limits and the diploid number of chromosomes is constant. The conditions in the cambium do not support Winkler's view that there is a close correlation between chromosomal number (chromosomal mass) and cell size in the somatic tissue of plants, and that giant cells are hyperchromatic.

4. The process of cell plate formation in the cambium is a remarkable phenomenon, and one which is significant in discussing the relative merits of various theories concerning the dynamics of karyokinesis and cytokinesis.

5. The newly formed partition membranes in the cambial initials frequently intersect the side walls at angles of varying degrees of acuteness, which is in contradiction to Errera's (Plateau's) Law of Minimal Area.

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EXPLANATION OF PLATE 1.

FIG. 1. Scale drawing, showing the relative sizes of ray initials and long initials in the cambium of a 60 year old stem of *Pinus strobus* L. $\times 100$.

FIG. 2. Scale drawing, showing the relative sizes of ray initials and large initials in the cambium of a year old stem of *Pinus strobus*. $\times 100$.

FIG. 3. Nucleus from large initial shown in Fig. 1. $\times 1,000$.

FIG. 4. Nucleus from large initial shown in Fig. 2. $\times 1,000$.

FIG. 5. Nucleus from ray initial shown in Fig. 1. $\times 1,000$.

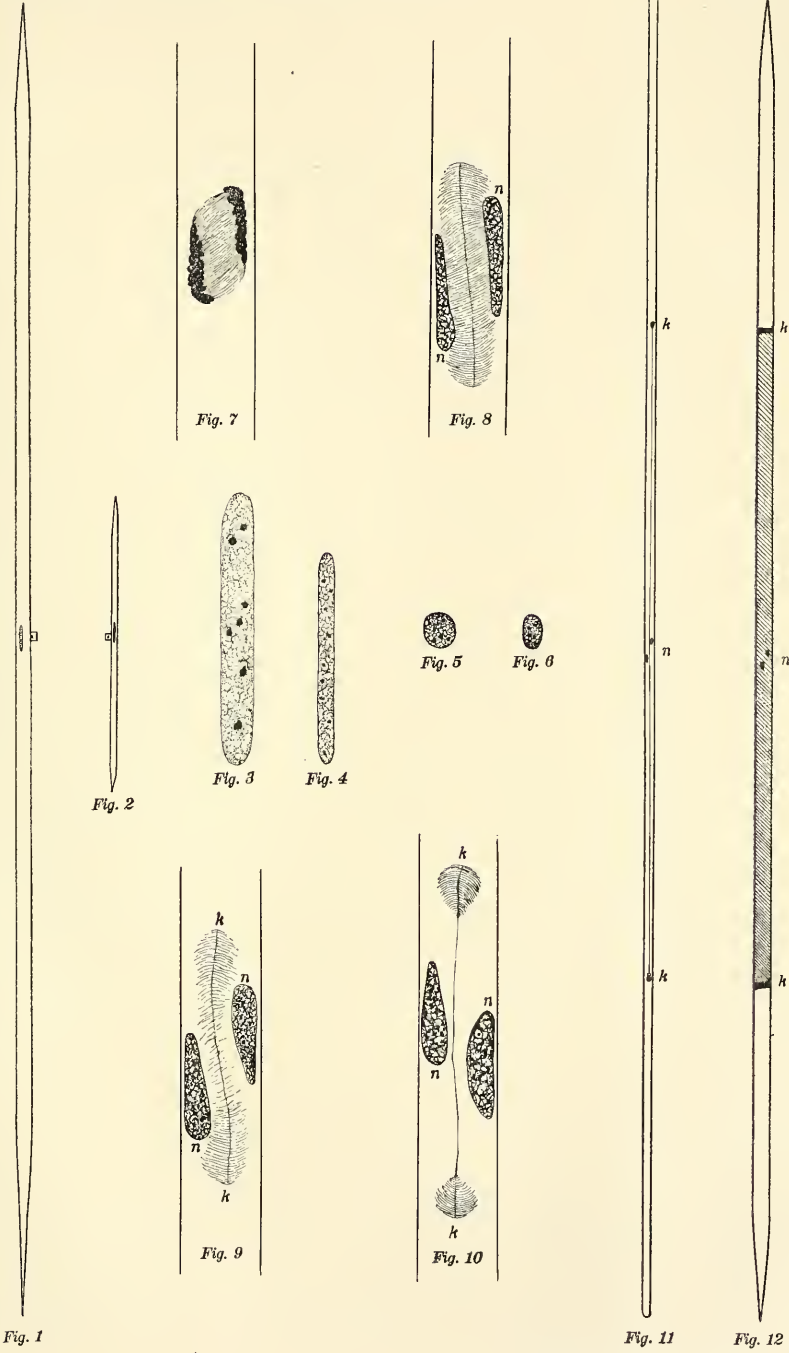
FIG. 6. Nucleus from ray initial shown in Fig. 2. $\times 1,000$.

FIG. 7. Karyokinetic figure in longitudinally dividing cambial initial of *Pinus strobus*. $\times 3,000$.

FIGS. 8, 9, and 10. Early stages in the formation of kinoplasmasomes and cell plate; (*n*) daughter nuclei, (*k*) kinoplasmasomes. $\times 3,000$.

FIG. 11. Radial longitudinal extension of cambial initial, showing later stage in the formation of the cell plate. $\times 100$.

FIG. 12. The same. Tangential longitudinal extension. $\times 100$.



(Bailey: Significance of cambium)

A QUANTITATIVE STUDY OF THE EFFECT OF ANIONS ON THE PERMEABILITY OF PLANT CELLS.

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(Received for publication, January 25, 1920.)

Although the effects of cations on the permeability of plant cells have frequently been studied, comparatively little attention has been paid to the action of anions.

The writer has investigated the effects of a series of anions on the permeability of *Laminaria agardhii* Kjellm. Measurements were made of the electrical conductivity by the method described by Osterhout.¹

The salts were the purest obtainable and the distilled water was not toxic to delicate test objects. The salt solutions were neutral to phenolsulfonephthalein (pH 7 ± 0.5). The temperature during the course of the experiments was $19 \pm 2^\circ\text{C}$.

Sodium sulfocyanide, iodide, nitrate, bromide, chloride, acetate, sulfate, tartrate, phosphate, and citrate were selected for study.

Solutions of these salts were made of the same conductivity as a slightly diluted sea water solution; *viz.*, a solution having the same conductivity as a 0.51 M solution of sodium iodide. (Normal sea water has the same conductivity as a 0.57 M solution of sodium iodide.) As is well known, various samples of sea water differ slightly in their conductivity and hence no absolute concentration can be given for each salt. The following table shows the approximate concentrations used.

Salt	SCN	I	NO ₃	Br	Cl	Acetate.	SO ₄	Tartrate.	PO ₄ *	Citrate.
M	0.62	0.51	0.59	0.51	0.45	1.1	0.36	0.59	0.35	0.43

*The phosphate is a mixture of 0.23 M disodium phosphate and 0.12 M monosodium phosphate.

¹ Osterhout, W. J. V., *J. Biol. Chem.*, 1918, xxxvi, 557.

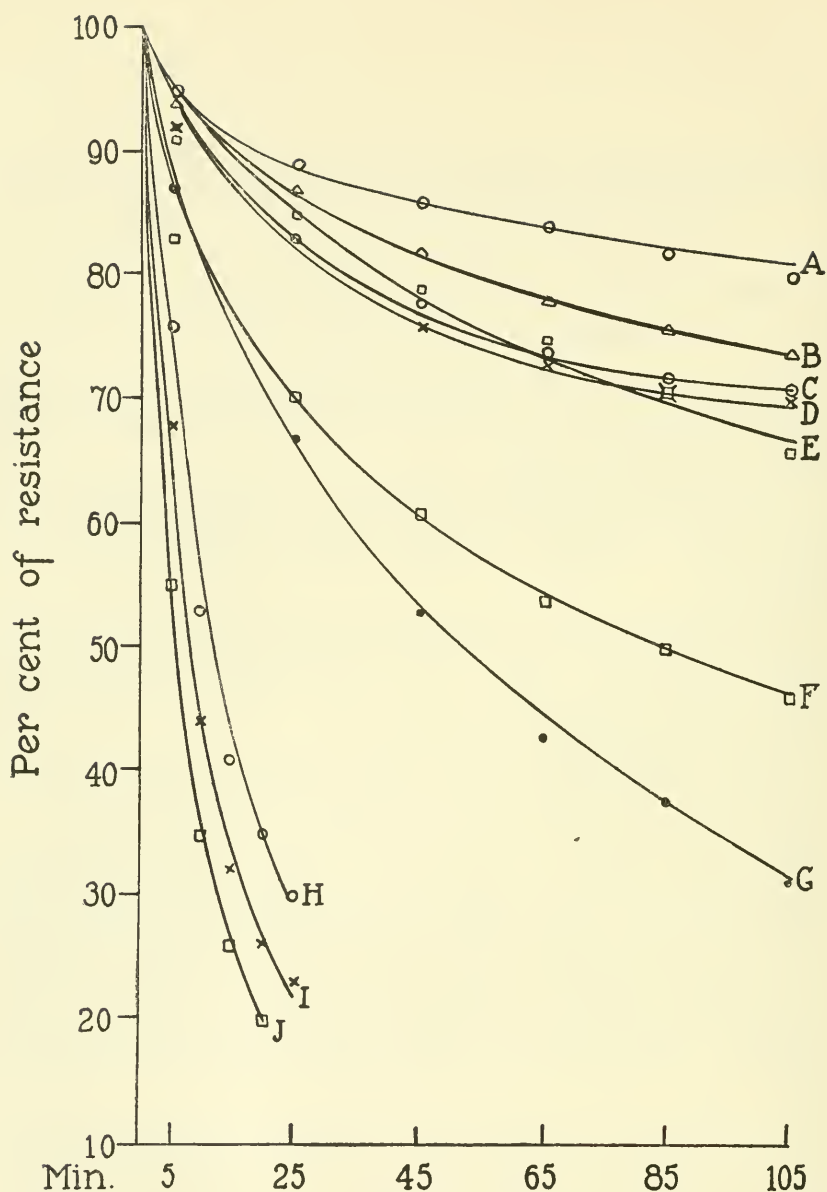


FIG. 1. Curves showing the resistance of *Laminaria* in solutions of various sodium salts: A in iodide; B in bromide; C in sulfocyanide; D in chloride; E in nitrate; F in acetate; G in sulfate; H in tartrate; I in phosphate; J in citrate. Ordinates represent resistances (expressed as per cent of the resistance in sea water, which is taken as 100 per cent). Each point represents the average of ten experiments. Probable error less than 10 per cent of the mean.

Measurements of the conductivity (permeability) were taken at the end of 5, 25, 45, 65, 85, and 105 minutes in the salt solution except in certain solutions (citrate, phosphate, and tartrate) where the resistance diminished very rapidly.

The results are shown in Fig. 1, the curve for each salt representing an average of ten experiments. The probable error of the mean (as based on Peter's formula) is always under 10 per cent, and for 75 per cent of the points is under 5 per cent.

It will be noted that the effect of these anions upon the increase in permeability as seen at the end of the experiment is in the series I, Br, SCN, Cl, NO₃, acetate, SO₄, tartrate, PO₄, citrate.

This differs from the Hofmeister series in that the effect of SCN is noticeably more than that of the iodide while the two effects were indistinguishable by Hofmeister, and also in that the relative positions of chloride and nitrate are reversed in this work from that found by both Pauli and Hofmeister. That these positions, however, are not invariable has been shown by Höber who obtained so called "transition series" by varying the hydrogen ion concentration² and the concentration of the salt.³ It may be noted, however, that at the end of the 25, 45, and 65 minute periods, when the disturbance affecting the experiment at the start has subsided and before possible disturbing factors due to the purely mechanical deterioration of the tissue have appeared, the position of the nitrate is above that of the chloride.

It is also seen that there is a distinct grouping into two or three groups. The iodide, bromide, sulfocyanide, chloride, and nitrate behave very similarly, while the acetate, sulfate, tartrate, phosphate, and citrate have a much more striking effect. In order to have solutions of the same conductivity as the sea water used, it was found necessary, as already mentioned, to employ different concentrations. But it is also desirable to make a comparison on the basis of the same molecular concentration. Since the concentration of two of the salts was close to 0.52 M and since this is the concentration of sodium

² Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipsic, 4th edition, 1914, 323.

³ Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipsic, 4th edition, 1914, 324.

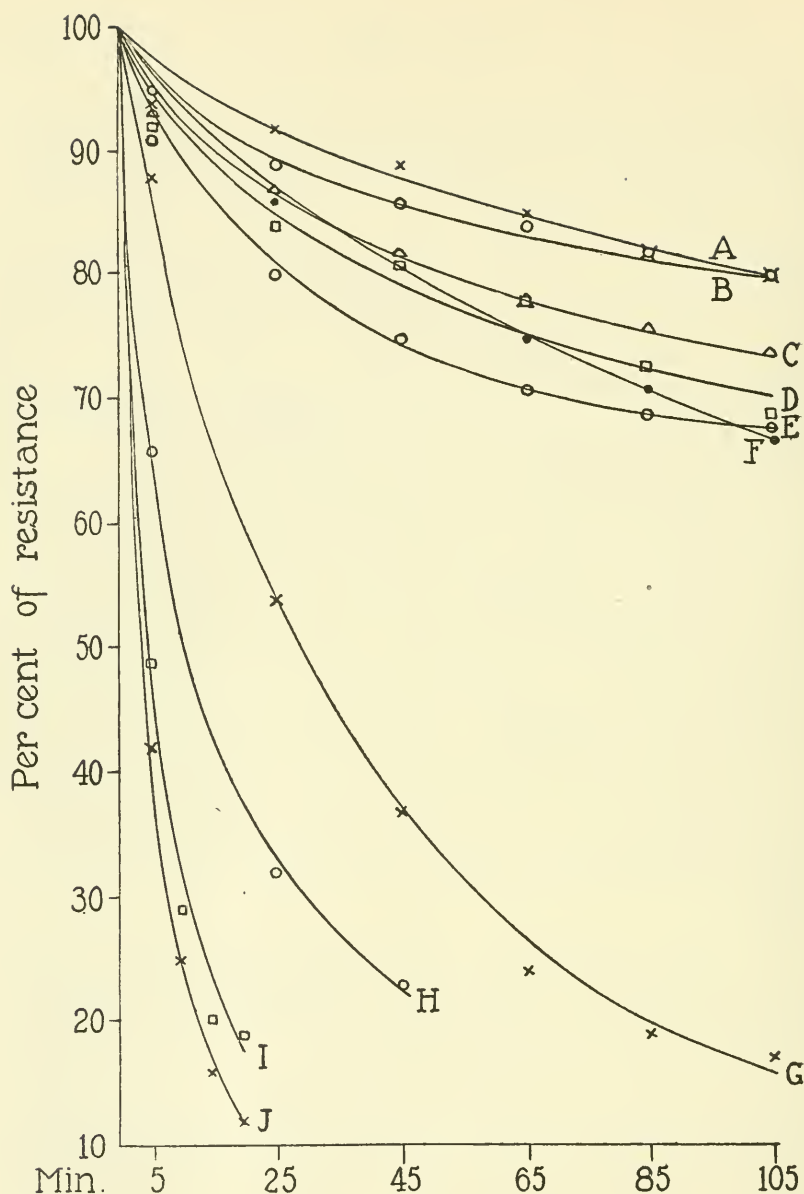


FIG. 2. Curves showing the resistance of *Laminaria* in solutions of various sodium salts: A in sulfocyanide; B in iodide; C in bromide; D in nitrate; E in chloride; F in acetate; G in sulfate; H in tartrate; I in phosphate; J in citrate. Ordinates represent resistance (expressed as per cent of the resistance in sea water, which is taken as 100 percent). Each point represents the average of ten experiments. Probable error less than 10 per cent of the mean.

chloride required to produce a solution of the same average conductivity as undiluted sea water, this was the molecular strength decided upon.

Sea water was concentrated (or diluted) in each case until it possessed the same conductivity as the solution to be tested. After the tissue had been placed in the concentrated (or diluted) sea water long enough to come to equilibrium (20 to 30 minutes) a measurement of the resistance was taken and the material then placed in the salt solution after which the procedure was the same as in the previous experiments.

Fig. 2 shows the results of these experiments. The results for each concentration of each salt are the average of ten experiments as before, all the concentrations being 0.52 M, with the exception of that of the phosphate. In this case the solubility of the disodium salt prohibits a concentration higher than 0.26 M; enough of the monosodium salt was added to produce neutrality and the total molecular concentration reckoned on this basis was about 0.39 M.

In this series the sulfocyanide takes its place at the top along with the iodide, and at the end of the experiment the two were identical. The nitrate curve is raised a trifle so that it now comes between the bromide and the chloride. The chloride, phosphate, and citrate drop slightly and the tartrate rises slightly, all of which changes are to be expected.

The greatest changes occur in the sulfate and acetate, which were farthest from 0.52 M in the first set of experiments. The acetate rises and takes its place among the other monovalent salts and the sulfate drops towards the tartrate and the trivalent salts, thus forming two distinct groups. It should be noted that the acetate comes from Group II up to Group I, but that its rate of fall at the end of the experiment was greater than for the others.

Several explanations for these phenomena may be given, but it does not seem worth while to enter upon a discussion of them at present. The recent work of Loeb on the relation between gelatin and electrolytes⁴ may furnish a clue to the ultimate explanation.

⁴Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 39, 237, 363, 483, 559.

THE ANTAGONISTIC ACTION OF ANIONS.

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(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, February 10, 1920.)

Striking cases of antagonism among cations are well known but the search for similar relations among anions has achieved little result. Some cases have been described by Loeb¹ and Miss Moore.² Lipman and his associates³ have reported antagonistic action of anions as the result of studies on bacteria in which salts were added to the soil, but it is very difficult to separate the effects of the added salts from those already present in the soil. Miyake⁴ found some antagonism among anions in studying the growth of wheat. Fenn⁵ has called attention to the fact that this kind of antagonism is commonly met with in experiments on gelatin.

The writer has observed some cases of this kind of antagonism among plants. A quantitative study of one of these cases is here presented. This study was carried on at Cambridge in the fall of 1919. The marine alga, *Laminaria agardhii* Kjellm was employed, the material being shipped from Woods Hole to Cambridge as needed. The material when it arrives in the laboratory is known to be in good condition since its resistance can be measured and compared with that of freshly gathered material. When kept at a low temperature the alga usually maintains its original resistance and vitality for several days. It was usually shipped in a covered firkin containing sea water. The firkin was surrounded by ice and was water-tight so that water from the melting ice could not dilute the sea water.

¹ Loeb, J., *Arch. ges. Physiol.*, 1905, cvii, 252 and literature cited there.

² Moore, A., *Am. J. Physiol.*, 1901, v, 87; 1902, vii, 315.

³ Lipman, C. B., *Centr. Bakteriolog., 2te Abt.*, 1912-13, xxxvi, 382. Lipman, C. B., and Burgess, P. S., *Centr. Bakteriolog., 2te Abt.*, 1914, xli, 430; 1914-15, xlii, 502. Lipman, C. B., *Plant World*, 1914, xvii, 295.

⁴ Miyake, K., *J. Coll. Agric. Tohoku Imp. Univ.*, 1914, v, 241; *J. Biol. Chem.*, 1913-14, xvi, 235.

⁵ Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiv, 141.

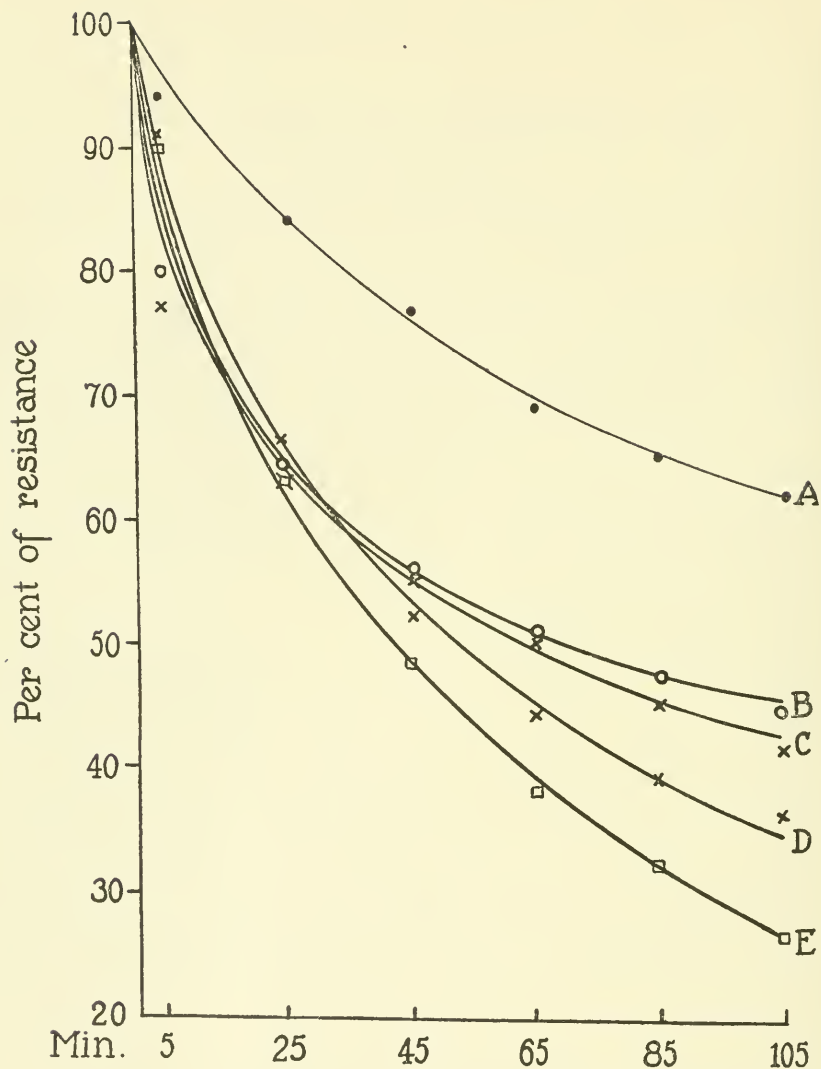


FIG. 1. Curves showing the resistance of *Laminaria* in 1.1 M sodium acetate, in 0.36 M sodium sulfate, and in mixtures of both: A in equal parts (by volume) of acetate and sulfate; B in acetate 75, sulfate 25; C in acetate; D in acetate 25, sulfate 75; E in sulfate. Ordinates represent resistance (expressed as per cent of the original resistance in sea water which is taken as 100 per cent). Each point represents the average of ten experiments: probable error of the mean less than 5 per cent.

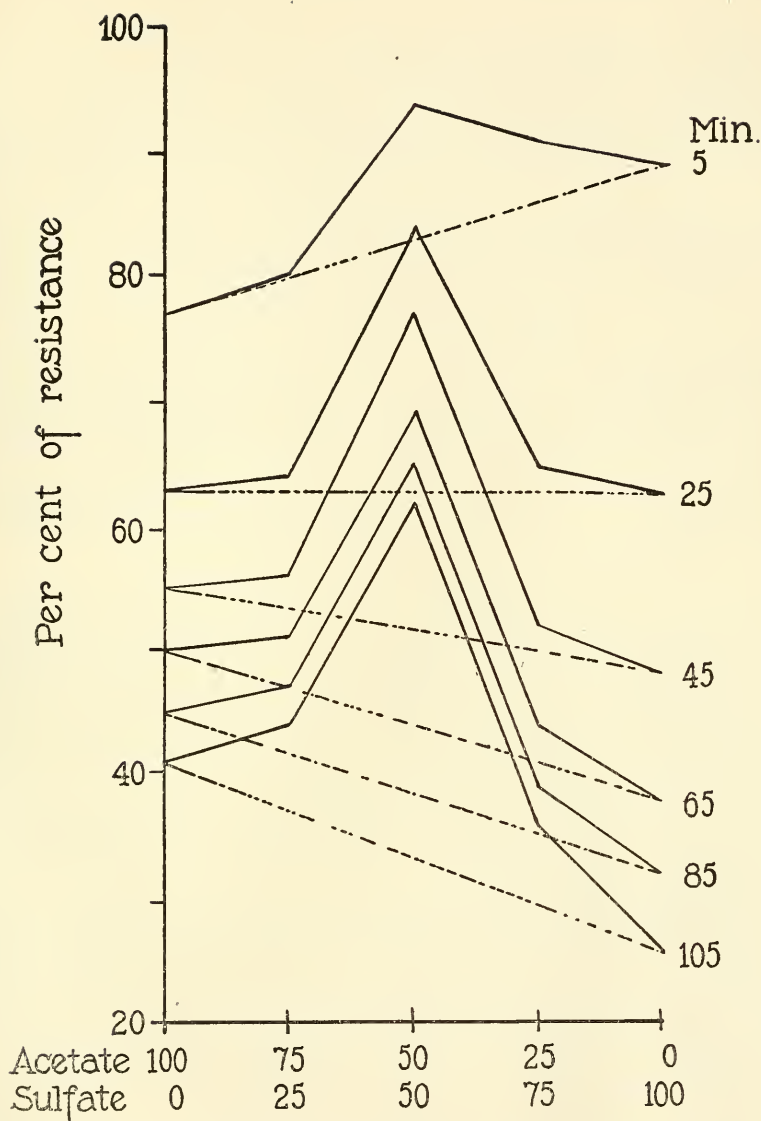


FIG. 2. Antagonism curves showing the resistance of *Laminaria* in 1.1 M sodium acetate, in 0.36 M sodium sulfate, and in mixtures of both. Ordinates represent resistance (expressed as per cent of the original resistance in sea water which is taken as 100 per cent); abscissæ represent volumetric proportions of the two salts. The dotted line connecting the ends of each curve shows the approximate additive effect; the vertical distance of the curve above this dotted line may be regarded as a measure of antagonism.

The method employed to measure the effects of the salts is the same as that mentioned in a former paper.⁶

The salts used were the purest obtainable. The distilled water was not toxic to delicate test objects. The solutions had a pH value of 7 ± 0.5 as measured by phenolsulfonephthalein and buffers which had previously been checked by the gas chain method. The experiments were conducted at a temperature of $19 \pm 2^\circ\text{C}$.

This paper deals with the effects of sodium acetate and sodium sulfate. Solutions of these salts were made of the same electrical conductivity as a slightly diluted sea water (about 1.1 M for sodium acetate and about 0.36 M for sodium sulfate) and the resistance of the tissue in solutions of the pure salts and in mixtures was measured after 5 minutes and thereafter at intervals of 20 minutes.

Fig. 1 shows the results. Each curve represents the average of ten experiments. The probable error of the mean (as computed by Peter's formula) is under 5 per cent in all cases.

On placing tissue in the pure acetate we observe that at the end of $1\frac{3}{4}$ hours the resistance has fallen to about 40 per cent of the original and in the pure sulfate it has fallen to about 25 per cent of the original, while in the mixture composed of equal volumes of the solution of each salt the resistance has fallen only to about 60 per cent. If no antagonism were present, the resistance of the mixture should drop to about 35 per cent (additive effect).

Fig. 2 shows the antagonism curves after various intervals using resistance for ordinates and salt proportions as abscissæ. Here the antagonism is clearly evident.

In view of the importance of the subject, further investigation seems desirable. The case here described is so striking as to warrant the conclusion that any theory of the antagonistic action of salts must take account of anions as well as of cations.

⁶ Raber, O. L., *Proc. Nat. Acad. Sc.*, 1917, iii, 682.

THE NATURE OF THE GROWTH RATE.*

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(From the University of California Graduate School of Tropical Agriculture and Citrus Experiment Station, Riverside.)

(Received for publication, March 5, 1920.)

Recent studies (Robertson (1908) and Reed and Holland) have shown that the rate of growth of certain organisms corresponds to the rate of an autocatalytic reaction. At the present time it seems profitable to extend the inquiry to several different types of plants and to learn something of their growth rates.

The Growth Rate of Pear Shoots.

During the season of 1919, weekly measurements were made of the growth of shoots of young Bartlett pear trees. The young shoots of the pear (*Pyrus communis* L.) are good material for such inquiry because of their vigorous growth and generally unbranched condition during the 1st year. The trees on which the shoots were selected and marked had been planted 3 years previously. They made good growth during the season in which the measurements were being made, but, on account of their age, produced no fruit. On April 24, 1919, fifty shoots on twenty-six trees in different parts of the orchard were selected. One shoot was subsequently broken and had to be eliminated. The remaining forty-nine shoots were measured each week until September 3, at which time elongation had ceased. The length of each new shoot was determined weekly by measuring the distance from its base to the apical bud. Though a few shoots did not cease elongation until the 133rd day (September 3) the average length of the growing season of all shoots was 97 ± 1.7 days.

* Contribution No. 65 from the University of California Graduate School of Tropical Agriculture and Citrus Experiment Station.

Robertson has called attention to the correspondence between growth of organisms and autocatalysis. He has shown that the rate of growth may also be expressed by the differential equation

$$\frac{dx}{dt} = kx (1 - x)$$

in which x is the size of the organism at time t ; a is the final size of the organism; and k is a constant. When integrated, the above equation becomes

$$\log \frac{x}{a - x} = K(t - t_1)$$

where $K = ak$, t_1 is the time at which the organism has reached half its final size; *i.e.*, the time when $x = \frac{a}{2}$. With the assistance of tables

which Robertson (1910-15) has prepared, the constants and the theoretical values of x are easily obtained from observational data. The value of a was taken as 114 cm. and t_1 as 47.4 days. Table I shows the computations made and the several values of K . With the average value of K the values of x were calculated by the formula and are given in the fourth column of Table I. The agreement between the two is good except for the first three measurements. Fig. 1 shows the curve obtained from the calculated values together with the observed values of mean length at weekly intervals during the growing season.

This growth rate is of considerable physiological interest if we compare it with the growth rates of organisms mentioned elsewhere in the literature. Attention may be called to the fact that we are here dealing with the mean growth of a number of separate shoots on a small population of trees.

A word concerning the heterogeneity of the population may be useful. On the final day of measurements the shoots ranged in length from 69 to 171 cm. with an average of 113 cm. The standard deviation of the measurements was 21.14 cm. This indicates that we are dealing with a random selection of material and not one which was so closely selected that it was not representative of the shoots of such trees.

TABLE I.
Growth Rate of Bartlett Pear Shoots for the Season of 1919.

<i>t</i>	<i>x</i> (observed).	<i>K</i>	<i>x</i> (calculated).
<i>days</i>	<i>cm.</i>		<i>cm.</i>
0	0		8.1
7	5	0.0331	10.7
14	11	0.0292	15.4
21	19	0.0264	21.3
28	29	0.0241	28.8
35	40	0.0215	38.1
42	48	0.0256	48.5
49	59	0.0194	54.4
55	69	0.0243	69.0
63	76	0.0194	80.4
70	86	0.0215	88.8
77	94	0.0228	95.6
84	100	0.0233	100.9
91	102	0.0214	104.8
98	108	0.0247	107.6
105	110	0.0250	109.6
112	111	0.0244	110.9
119	112	0.0243	111.9
126	113	0.0260	112.6
Average		0.0242	

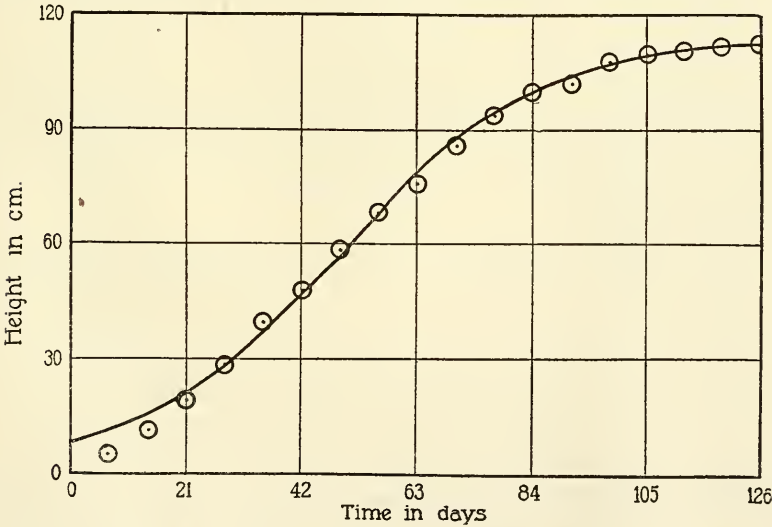


FIG. 1. Growth rate of shoots of the pear tree. The curve represents the value obtained from the equation $\log \frac{x}{114-x} = K(t-47.4)$; points within the circles represent observed lengths at intervals of 7 days.

The data suggest that each shoot has a more or less independent supply of the catalytic agent which exercises such an important influence upon the rate at which growth proceeds.

The Growth Rate of Young Walnut Trees.

The data upon which this study is based were obtained from material growing in the grounds of the Citrus Experiment Station at Riverside.

In the spring of 1916 seeds of *Juglans nigra* L. (black walnut) had been planted in rows for nursery propagation work. The young trees which grew from these seeds were used for study in the summer of 1917; *i.e.*, during their second season of growth. A part of the trees were grafted in the spring of 1917 with scions of *Juglans regia* (Persian walnut). A few weeks after growth from the scions started, all new shoots were removed, except one which furnished the data for the observations on this species. The balance of the *Juglans nigra* trees grew on during the second summer.

The *Juglans nigra* seedlings were selected on April 17 and marked by a line of India ink on the stem from which the distance to the terminal bud could be measured when desired. From a portion of the trees all laterals on the wood of the previous year were removed before measurements were begun, and the observations on these pruned trees were separately recorded. The growth from the *Juglans regia* scions was soon sufficient to afford measurements, and they were marked on May 29.

Measurements were made at each day of observation to determine the height of the tree stem from the ink mark to the terminal bud of the main axis. This could be ascertained with comparatively little error. It was planned to make measurements at intervals of 7 days, but other circumstances made it necessary to vary this interval at times. The exact length of the intervals is stated in Table II.

The population thus gave an opportunity for studying the following points: (a) the growth of seedling trees in the 2nd year of their life; (b) the growth of similar trees from which all laterals had been removed at the beginning of the 2nd year; and (c) the growth from scions of a distinct species growing on the same roots as (a) and (b).

Group (a) contained nineteen trees, Group (b) fifteen trees, and Group (c) twenty-five trees.

The mean height of the selected trees on the several days of measurement is represented graphically in Fig. 2. The data show that *Juglans nigra* grew quite uniformly from the commencement of the measure-

TABLE II.
Mean Height of Walnut Trees.

Days from Apr. 17.	Mean height of <i>Juglans nigra</i> trees. Laterals not removed.	Mean height of <i>Juglans regia</i> trees on <i>Juglans nigra</i> roots.
	cm.	cm.
0	—	—
7	56.2 \pm 1.92	
14	61.2 \pm 1.73	
21—	64.4 \pm 2.37	
28	69.1 \pm 2.68	
34	71.5 \pm 2.71	
41	73.5 \pm 2.82	
48	77.0 \pm 2.99	
55	79.1 \pm 3.03	63.24 \pm 0.55
62	80.9 \pm 3.14	67.80 \pm 0.66
69	84.6 \pm 3.23	74.88 \pm 0.95
84	105.9 \pm 3.23	102.6 \pm 2.46
87	109.6 \pm 3.70	110.2 \pm 2.65
96	117.6 \pm 4.01	126.6 \pm 3.47
103	127.6 \pm 4.55	140.6 \pm 3.97
110	130.3 \pm 5.01	152.0 \pm 4.25
117	131.8 \pm 5.25	162.4 \pm 4.78
131	132.9 \pm 5.15	176.0 \pm 5.30
138	134.5 \pm 5.65	184.0 \pm 5.76
144		188.8 \pm 5.82
151		195.2 \pm 6.35
158		198.4 \pm 6.47
165		199.2 \pm 6.67
172		200.0 \pm 6.87
179		200.8 \pm 6.80

ments on April 17 until about June 19 (the 62nd day). Between June 19 and August 6 (between the 62nd and 110th days) growth was considerably more rapid, but after August 6 growth gradually diminished until it ceased about September 3.

The mean growing period of this group of trees was 124 days after April 17. The coefficient of variability of the population at each

time of measurement had an approximate value of 25. There are fluctuations from this value, but the trees showed little tendency to increase in variability until near the end of the growing period.

The *Juglans nigra* trees from which all laterals were removed in the spring before growth began made the same type of growth as their unpruned neighbors, but in every case their mean height was less. The commonly accepted belief is to the effect that removing the lat-

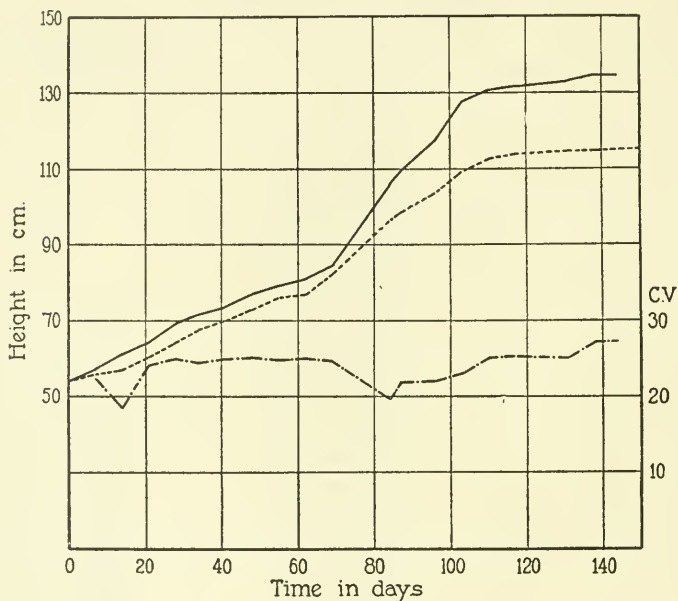


FIG. 2. The growth of *Juglans nigra* trees. ——— increase in mean height of unpruned trees. - - - - increase in mean height of trees from which laterals had been removed. — . — . — Coefficient of variability of mean height of unpruned trees.

eral branches from a plant increases the growth of the main axis, but the results of this work directly contradict the above mentioned belief.

The mean growing period of the pruned seedlings was 122 days, or practically the same as that of the unpruned seedlings. Comparison of the figures shows that the pruned seedlings increasingly lagged behind their unpruned neighbors as the season advanced.

The *Juglans regia* trees were measured from June 5 to October 22. The data afforded by these measurements are shown in Table II. The average length of their growing season was 116 days from June 5, which means that the average tree ceased to grow on September 28, though it was October 22 before all trees ceased to increase in height.

We may proceed to examine the growth rate of these trees by a method which has elucidated many problems in the domain of physical chemistry.

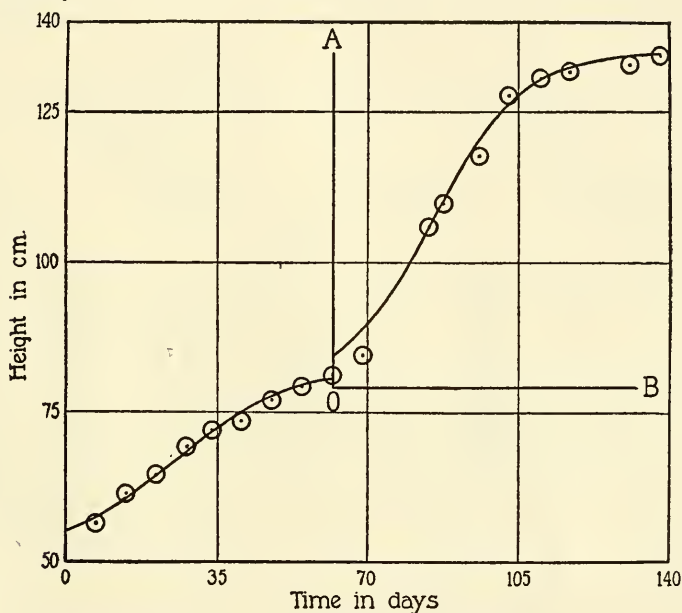


FIG. 3. Growth rate of *Juglans nigra* trees showing two cycles. Curves show computed values; points within circles show observed mean height at various intervals. AOB are axes for the upper curve.

Reference to the graph representing the mean height of the trees at successive intervals shows that the curve consists of two rather distinct parts, indicating that the growth period comprised in these records consisted of two cycles. The curve rises rather regularly from the 1st to the 62nd day, then rises more steeply to near the 110th day, when it takes a nearly horizontal position. A few simple calculations will show that the growth curve does not follow the path of a single autocatalytic reaction.

The period was therefore divided into two cycles, the first of which ended at the 62nd day, and the second of which began at approximately the time the first cycle ended. Reference to Table III will show the computations made. Since we are interested in rate it will be proper to move our base line up to a point near that at which the first cycle actually started. Actually 50 cm. were subtracted from each mean in order to give a curve which would start near the inter-

TABLE III.

Height of Juglans nigra Trees. Comparison of Observed Values with Those Computed from the Formula $\log \frac{x}{a-x} = K(t-t_1)$.

First cycle.				Second cycle.			
<i>t</i>	$x - 50$ (observed).	<i>K</i>	$x - 50$ (calculated).	<i>t</i>	$x - 79$ (observed).	<i>K</i>	$x - 79$ (calculated).
<i>days</i>	<i>cm.</i>		<i>cm.</i>	<i>days</i>	<i>cm.</i>		<i>cm.</i>
7	6.2	0.0375	7.2	62	1.9	0.0632	5.4
14	11.2	0.0283	10.5	69	5.6	0.0596	9.7
21	14.4	0.0348	14.5	84	26.9	0.0350	26.7
28	19.1	0.0380	18.7	87	30.6	0.0400	30.7
34	21.5	0.0296	22.0	96	38.6	0.0313	41.7
41	23.5	0.0252	25.2	103	48.6	0.0454	47.7
48	27.0	0.0299	27.6	110	51.3	0.0415	51.5
55	29.1	0.0317	29.2	117	52.8	0.0381	53.6
62	30.9	0.0374	30.3	131	53.9	0.0308	55.4
				138	55.5	0.0385	55.7
Average		0.0325		0.0423

section of the abscissa with the ordinate. For the first cycle the value of a was taken as 32, and t_1 as 23.5. Accordingly

$$\log \frac{x}{32-x} = K(t-23.5)$$

From this, the several values of K were computed and found to be fairly uniform. The average value of K was taken as 0.0325, and from it the values of x were computed by the above formula. The root-mean-square deviation $\left(\sqrt{\frac{d^2}{n}}\right)$ from the observed values was only 0.78 cm. It will thus be seen that the agreement between observed and calculated values is good.

The data for the second cycle were similarly treated. The values were all diminished by 79 cm. The value of a was taken as 56 and that of t_1 as 85. The mean value of K was determined to be 0.0423 and from this the values of $x-79$ were calculated by the same general formula as for the other cycle. The agreement between the calculated and observed values is quite good. A graphical representation of the values is shown in Fig. 3.

It thus appears that between April 17 and September 4 the growth of this population of *Juglans nigra* trees fell into two distinct cycles and that the rate of growth for each cycle followed closely the course of an autocatalytic reaction.

A similar type of growth rate was also found for the *Juglans nigra* trees from which the laterals had been removed at the beginning of the season. It has been shown (Reed and Holland) that the smaller members of a population have a type of growth rate similar to that of the larger individuals. That is, both groups were found to correspond to the equation which states that the growth in unit time is proportional to the product of the height at that time and the amount yet to grow, multiplied by a constant.

The growth rate of the *Juglans regia* trees was likewise studied. Here again it is advisable to deduct a constant amount from each height measurement in order to bring the base line near the point of origin of the part of the growth curve studied. In this case $x_1 = x - 50$. The value of a was found by approximations to be 153.2 cm. and $t_1 = 96$. We therefore have

$$\log \frac{x}{153.2 - x_1} = K(t - 96)$$

Table IV shows the observed values of x_1 , the various values of K , and the calculated values of x_1 with the mean value of K . The root-mean-square deviation is 3.5 cm. The smallness of this divergence becomes apparent when we note that the probable errors of the mean values of x (Table III) are greater than 3.5 except in the first six cases. A graphic representation is given in Fig. 4.

It will be noted that the data for the growth rate of *Juglans regia* extend from June 5 to October 15. There appears to be but one growth cycle between these dates. This period begins close to the

time at which *Juglans nigra* began its second growth cycle. We do not know whether *Juglans regia* had two growth cycles, but the presumption is in favor of such an hypothesis.

Data such as these afford a new insight into certain phases of growth in the organic world. They show that the growth rate of these or-

TABLE IV.

Height of Juglans regia Trees. Comparison of Observed Values with Those Calculated from the Formula $\log \frac{x}{a-x} = K (t-t_1)$.

<i>t</i>	<i>x</i> ₁ (observed).	<i>K</i>	<i>x</i> (calculated).
<i>days</i>	<i>cm.</i>		<i>cm.</i>
55	13.2	0.0250	15.9
62	17.8	0.0261	22.1
69	24.9	0.0264	29.9
84	52.6	0.0233	53.2
87	60.2	0.0210	58.8
96	76.6		76.6
103	90.6	0.0229	90.5
110	102.0	0.0213	103.6
117	112.4	0.0210	115.1
131	126.0	0.0190	134.7
138	134.0	0.0201	138.0
144	138.8	0.0205	141.9
151	145.2	0.0229	145.1
158	148.4	0.0244	147.5
165	149.2	0.0228	149.2
172	150.0	0.0222	150.4
179	150.8	0.0216	151.2
Average		0.0228	

ganisms follows a course as definite and as predictable as that of a chemical reaction. On the basis of pure chance we should not expect often to find a group of individuals which should follow a definite rate of development more closely than the laws of random sampling would require.

The onset of dormancy in the two species of *Juglans* is worthy of notice. It should be remembered that the two lots of trees grew in adjoining rows in the nursery and received similar care as to irrigation,

cultivation, etc. Nevertheless the *Juglans regia* trees grew 42 days later than the *Juglans nigra* trees. It is well known that there is a great difference between these species in respect to distribution and resistance to cold weather. *Juglans regia* is reported to be a native of Persia, the Himalayas, and China, while *Juglans nigra* is a native of the eastern United States. The remarkable fact, as far as these

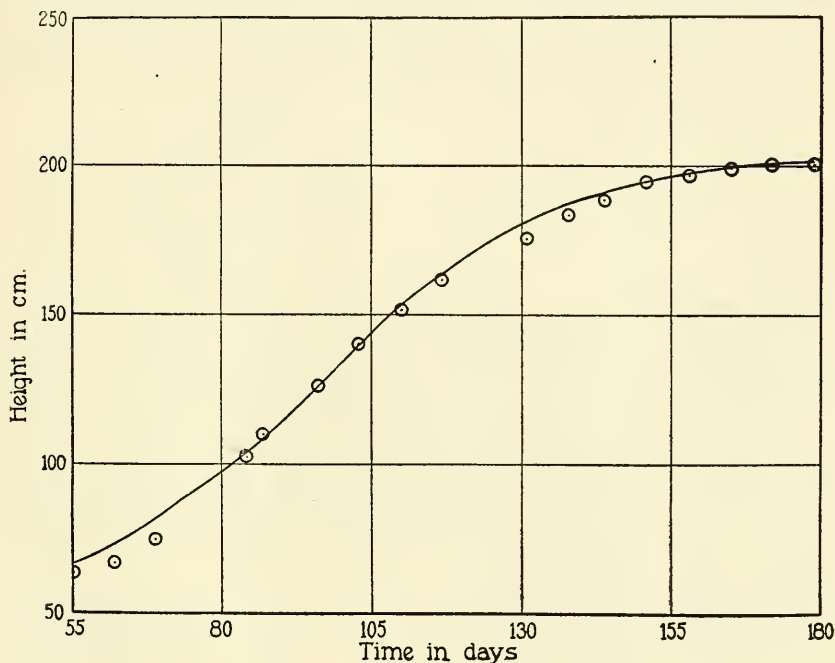


FIG. 4. Growth rate of *Juglans regia* trees. The curve represents the values obtained from the equation $\log \frac{x}{153.2 - x_1} = K(t - 96)$; points within circles represent the observed mean height at various intervals.

notes are concerned, is that *Juglans nigra* trees went into a condition of dormancy early in September, while *Juglans regia* trees, growing in adjoining rows and with similar care, grew until the middle of October.

This observation sustains the conclusion drawn from the foregoing studies on the rate of growth, and indicates that the extent of the growth cycle as well as the amount of growth is controlled by internal genetic factors.

The Reliability of Various Criteria for Determination of the Growth Rate.

It will be well to discuss certain questions which will arise in the mind of the reader. He desires to know, for example, whether the growth rate will conform to a differential equation when we use some other criterion, *e.g.* weight, as a measure of growth. An examination of all possible measures of growth should be made, especially in view of the statement sometimes heard that growth is a process so complex that no single equation can successfully represent it.

It should be noted at the outset that every measure which is a mean of separate determinations of mass or volume is accompanied by an inevitable error. This error is made up of two separate errors. The first is due to the fact that the individuals measured were not truly representative of the population, *i.e.* they did not constitute a good "random sample"; the second is due to the errors which arise in the use of the measuring device (meter stick or balances). If the errors are of a purely random nature they will largely offset one another in a large series, but in a small series they may materially affect the mean. We need not expect, therefore, to find agreement between observed and calculated values as close as the physical chemist obtains with his material. A difference of not more than 10 per cent of the calculated value may be regarded as sufficiently accurate to inspire confidence in the results.

Kreusler has recorded the growth of several varieties of maize at 7 day intervals. Height of plant, green weight (entire plant), and dry weight (entire plant) were determined on fairly large samples of plants. I have found that the growth rate of the different varieties determined by Kreusler follows the equation previously used

$$\frac{dx}{dt} = kx(a - x)$$

The determinations made upon one variety (Hühner-mais) will be given as an illustration. Those who are interested may profitably compute the growth rates of the other varieties which Kreusler followed. Reference to Table V shows undoubted errors in the weight of the samples at various dates. These can only be due to the use of too small a number of plants to give a true representation of the

population at that date. Table V shows a comparison of the growth rates of maize (Hühner-mais), with the height, green weight, and dry weight of the plants, recorded at intervals of 7 days. I have determined the value of K , the growth constant, for each measurement and, from the average value of all determinations, have calculated the value

TABLE V.

Rate of Growth of Maize.

Data taken from Kreusler (Hühner-mais).

<i>t</i>	Mean height.			Mean green weight.			Mean dry weight.		
	x (observed).	K	x (calculated).	x (observed).	K	x (calculated).	x (observed).	K	x (calculated).
<i>days</i>	<i>cm.</i>		<i>cm.</i>	<i>gm.</i>		<i>gm.</i>	<i>gm.</i>		<i>gm.</i>
7	8.0	0.0311	3.8						
14	12.7	0.0309	7.0				0.13	0.0536	0.09
21	17.4	0.0335	12.7	0.9	0.0665	1.1	0.19	0.0551	0.19
28	24.1	0.0374	22.2	2.5	0.0640	2.5	0.39	0.0547	0.43
35	36.4	0.0402	36.6	8.6	0.0605	7.2	1.10	0.0537	1.03
42	53.8	0.0472	55.5				2.97	0.0514	2.41
49	77.7	0.0453	76.2	53.4	0.0603	49.7	7.49	0.0486	5.59
56	99.0	0.0466	94.8	128.9	0.0454	111.5	15.37	0.0472	12.47
63	111.7	0.0441	108.7	193.4	0.0457	198.0	21.32	0.0688	25.03
70	124.0	0.0531	117.9	322.9	0.1119	278.0	43.13	0.0551	43.00
77	120.0	0.0339	123.2	304.2	0.0479	325.4	53.20	0.0301	60.97
84	127.7	0.0448	126.4	340.8	0.0703	346.7	79.63	0.0784	73.53
91	124.6	0.0297	128.1	353.8	0.0599	355.3	82.30	0.0641	80.41
Average		0.0398			0.0632			0.0551	

of x for corresponding values of t , using the tables prepared by Robertson (1910-15). The average divergence of the three sets of values is not large, particularly in view of the errors above mentioned. The results show a sufficiently good correspondence to inspire confidence in their reliability.

It will be seen that the growth rate of the maize plants follows the course of an autocatalytic reaction no matter whether we use as a criterion mean height, green weight, or dry weight. Their growth is similar to a chemical reaction in which one of the products of the reaction acts as a catalyst.

Attention may next be directed to some data recently published (Eckles and Swett) on the growth of heifers. The measurements

appear to have been carefully made and at regular intervals. They therefore afford good material for study. Eckles and Swett used various criteria for measuring the heifers. They concluded that height at the withers is the most reliable measure, though for comparative studies weight is also useful.

¶ In computing the growth rate of these animals I have used a slightly different equation; *viz.*, $\frac{dx}{dt} = k(a - x)$ which upon integration becomes $x = a(1 - e^{-kt})$.

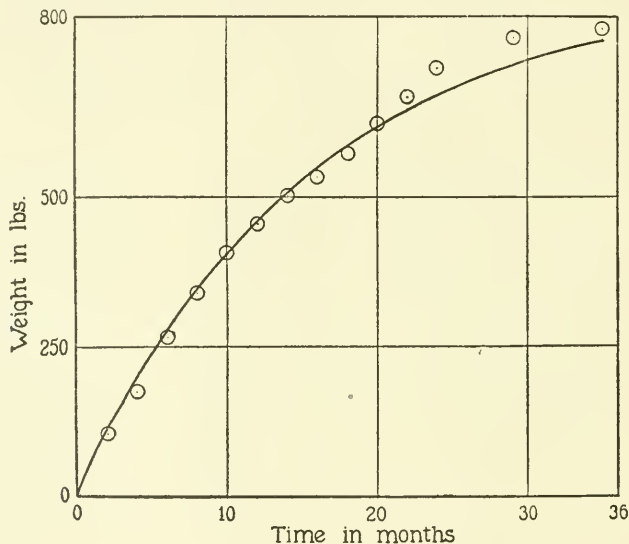


FIG. 5. \overline{P}_n Growth rate of Jersey heifers represented by mean weight. The curve shows the values obtained from the equation $x = 850(1 - e^{-0.065t})$; points within circles represent observed weights at intervals of 2 months.

The rate as thus expressed is directly proportional to the amount of growth yet to be made. It is therefore more rapid at the outset and slower near maturity than the formula used for computing the growth rate of maize in Table V. In the case of the heifers, a , the weight at 3 years of age, was found by a series of approximations to be 850 pounds, and k , the growth constant, was 0.065. The equation used for determining the calculated values of x was therefore $x = 850(1 - e^{-0.065t})$ (Fig. 5).

It will be seen that the observed and calculated values of x agree very well, especially when one bears in mind the extent of variability in biological material. In the latter part of the period the observed values are consistently higher than the calculated. These weight differences are plainly due to the increase in weight during pregnancy. The first parturition occurred at 30 months. As it is, however, the root-mean-square deviation between observed and calculated weights is only 20 pounds.

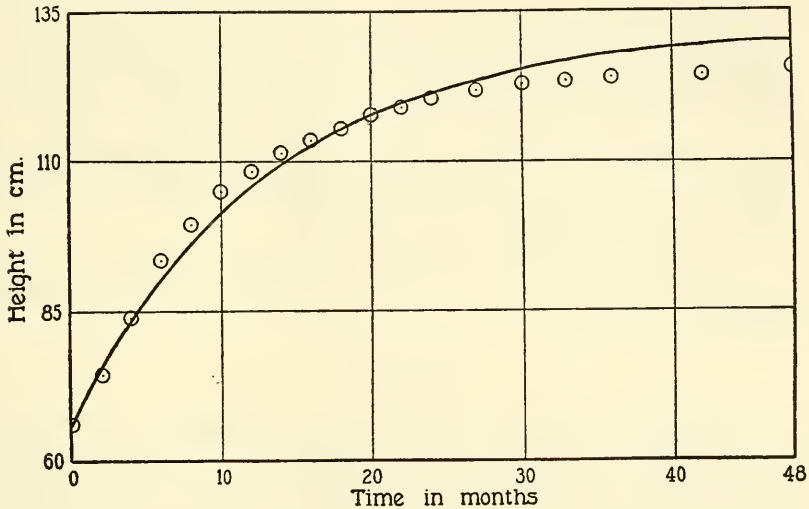


FIG. 6. Growth rate of Jersey heifers represented by height at withers. The curve shows the values obtained from the equation $x = 128 (1 - e^{-0.08 (t + 9)})$; points within circles represent observed heights at various intervals.

Inquiry may next be directed to the rate at which these animals increased in size and to the value of different kinds of measurements. Eckles and Swett found that the most reliable size measurement was the height of the animal at the withers. The mean height at birth was 66.1 cm.; at 4 years it was 125.6 cm. It thus appears that at birth the calf is a little more than half as tall as the mature cow. When we come to study the growth rate it is evident that we cannot use the postnatal life period as the complete growth cycle, because the animal has attained over half its height before birth. Let us assume that height growth begins near the beginning of the gestation period, then

we shall use $t+9$ as the time instead of t , the observed time from birth. By a series of approximations, 128 was taken as the value of a , and 0.08 as the value of k . The formula used was then $x = 128 (1 - e^{-0.08 (t+9)})$ (Fig. 6). The agreement for the most part is strikingly close. The close correspondence between the observed heights and those calculated by the formula bears out the observation of Eckles and Swett that the growth of the skeleton of the cow is less influenced by conditions of gestation and lactation than the other criteria of growth.

Summing up the data on the growth rate of Jersey heifers as given above, it appears that they grow by a definite rate which can be represented as a reaction in which the increase in any given interval is proportional to the amount of growth yet to be accomplished. It does not greatly matter whether one uses height at withers or weight, though the former is slightly more accurate.

In the case of maize and of heifers it appears that growth proceeds by a definite quantitative rate, no matter whether height or weight is used as a criterion.

SUMMARY.

1. The growth rate of organisms may be considered as a chemical reaction which gives the mature organism as its end-product. The organism grows at a definite rate which is, at any moment, proportional to the amount of growth yet to be made.

2. Shoots of young pear trees measured at weekly intervals during the growing season showed a rate similar to that of an autocatalytic reaction.

3. Young walnut trees showed distinct cycles of growth in a single season, but the growth in each cycle proceeded at a rate corresponding to an autocatalytic reaction.

4. The growth rate follows a definite, quantitative course though judged by different criteria. Data are presented for maize in which green weight, dry weight, and height of the plant are used. Data for cattle show that either weight or height of the animal may be used as a criterion.

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ON THE CAUSE OF THE INFLUENCE OF IONS ON THE RATE OF DIFFUSION OF WATER THROUGH COLLODION MEMBRANES. II.

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(Received for publication, February 28, 1920.)

I.

In this note it is intended to complete the proof that the influence of the concentration of electrolytes on the transport of water through a collodion membrane is similar in the case of free and of electrical endosmose.

Fig. 1 gives the curves representing the influence of different concentrations of KCl, K₂SO₄, K₃ citrate, and CaCl₂ upon the initial rate of diffusion of water from pure water through a collodion membrane into solution (free osmosis). The solution was inside a collodion bag and the latter dipped into a beaker filled with distilled water. The ordinates of the curves give the rise in the level of liquid after 20 minutes in a glass tube pushed through a rubber stopper into the collodion flask. The curves show, as pointed out in a preceding publication,¹ that the level rises at first with increasing concentration until it reaches a maximum at about M/512 or M/256 and that it then drops with a further rise in concentration until the latter is M/16 when a second rise begins. The second rise is presumably the expression of a prevalence of the gas pressure effect while the first rise and fall are the effect of the electrostatic influence of the ions on the rate of diffusion of water. As stated in the preceding publication, pure water as well as water containing electrolytes is positively charged when in contact with a collodion membrane while the latter is negatively charged. Leaving aside the gas pressure effect on the rate of diffusion, water will be driven through the pores or interstices of the

¹ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 173.

membrane from that side of the membrane which has a smaller density of charge to that side which has a greater density. The density of charge on a collodion membrane in contact with a watery solution is influenced in an opposite sense by the oppositely charged ions of an electrolyte in solution, the density being increased by that ion which

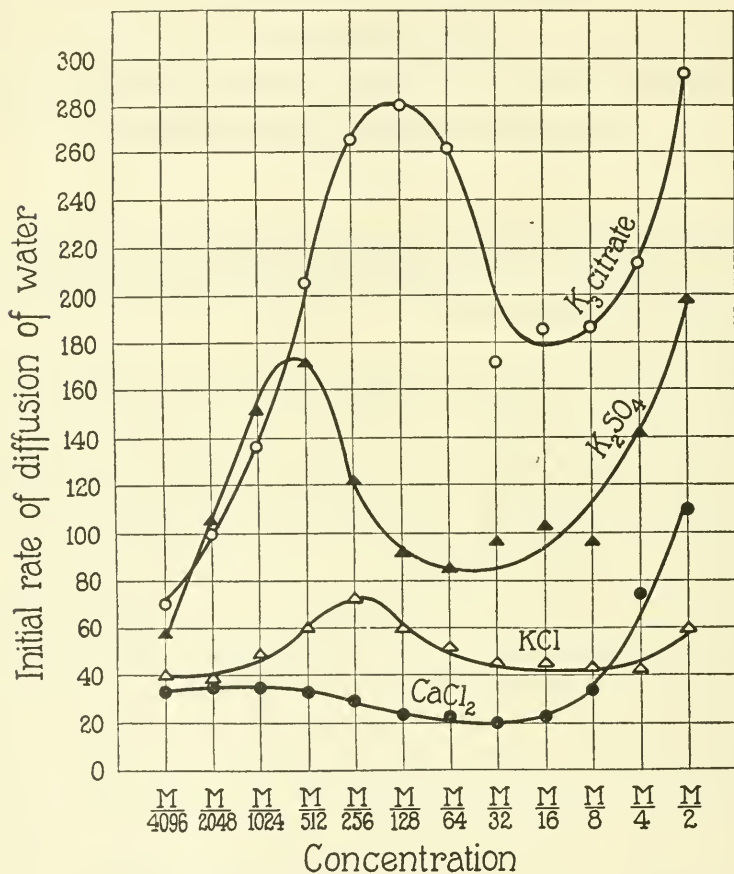


FIG. 1. *Free osmosis*. Effect of ions on free osmosis when water is positively charged. Inside the collodion bag, solutions of $CaCl_2$, KCl , K_2SO_4 , and K_3 citrate in different concentration; outside, H_2O . Abscissæ are the logarithms of concentrations of electrolyte in the collodion bag, ordinates the rise in level of liquid in manometer after 20 minutes (with the exception of K_3 citrate which is given after 10 minutes).

has the same sign of charge as the membrane and being diminished by that ion which has the opposite sign of charge.² Since the collodion membrane is negatively charged in contact with the solutions mentioned in Fig. 1, the density of charge on the membrane is increased by the anion and diminished by the cation of the electrolyte.

The relative influence of the oppositely charged ions of an electrolyte is, however, *not* the same for different concentrations. When the membrane is negatively charged the influence of the anion on the charge of the membrane increases in lower concentrations more rapidly with increasing concentration than the depressing effect of the cation until the concentration is $M/512$ or $M/256$. When this point is reached, the depressing influence of the cation on the negative charge of the membrane increases more rapidly with increasing concentration than the influence of the anion.

This explains why the curves representing the initial rate of diffusion of water from pure water through a collodion membrane to a solution of an electrolyte rise at first with increasing concentration until the concentration is about $M/512$ or $M/256$ and then generally drop with a further increase in concentration.³ The rise of the curves is higher the higher the valency of the anion and less the higher the valency of the cation. In the case of CaCl_2 the strong depressing effect of Ca prevents a rise by the Cl ion.

It follows from the theoretical discussion in the preceding paper² that if this explanation of the influence of the concentration of the electrolyte is correct, the rate of diffusion of water through a collodion membrane must vary in the same sense with the concentration of the electrolyte in the case of *electrical* endosmosis as it does in the case of *free* osmosis. This is shown to be true by the curves in Fig. 2. These curves represent the relative transport of water in the case of electrical endosmosis through a collodion membrane in the presence of the same electrolytes as those used in Fig. 1.

In these experiments the collodion flasks were filled with a solution of an electrolyte and were dipped into a beaker containing an identical solution. A large disc of platinum at the bottom of the beaker

² Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 387.

³ The second rise beginning with $M/16$ is due to the gas pressure effect and will not be discussed in this paper.

was one electrode while the other electrode was a platinum wire pushed through a glass tube into the collodion flask. The latter was closed with a rubber stopper through which the glass tube with a bore of 2 mm. diameter was pushed into the collodion flask. The distance between the two electrodes was 7.0 cm. A p.d. of 15 volts was applied and the current through the collodion membrane increased slowly in intensity until it finally became fairly constant. To accelerate this process a p.d. of 200 volts was used for 30 seconds or a little longer, and then the p.d. was changed to 15 volts. This, however, was done only in the case of the more dilute solutions. The

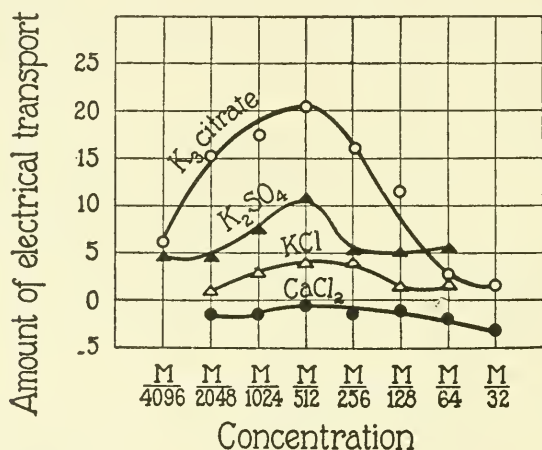


FIG. 2. *Electrical endosmose*. Effect of the same electrolytes as in Fig. 1 on electrical endosmose. Inside and outside the collodion bag identical solutions. Abscissæ are the logarithms of concentrations of electrolyte in solution, ordinates rise of level of liquid on the side of the cathode in 15 minutes. The influence of electrolytes on the rate of transport of water in electrical endosmose is similar to that in free osmosis (Fig. 1).

liquid rose in the glass tube when it contained the cathode, showing that the watery phase was positively charged. The rise in level in the glass tube was measured for 15 minutes (usually between 5 and 20 minutes after the commencement of the action of the current).

The reader will notice that the amount of liquid transported by the current rises at first with the increase in concentration of the solution until the latter is about $M/512$ and that with a further increase in

concentration the quantity of electro-endosmotic transport falls. The curves resemble those in Fig. 1 except that the maximum is a little lower in the case of electrical endosmose. The writer is inclined to explain this difference by the fact that in electrical endosmose the concentration of the liquid is increased by the secondary chemical reactions at the electrodes and that thereby in a M/512 solution of K_2SO_4 or KCl the concentration of the solution rises gradually as a consequence of electrolysis and secondary chemical reactions at the electrodes and approaches M/256.

It is, moreover, obvious that the electrical transport (Fig. 2) of the positively charged liquid increases with the valency of the anion as in free osmosis (Fig. 1) and that it diminishes with the increasing valency of the cation as shown by the flat curve for $CaCl_2$. The curves for $MgCl_2$ and $BaCl_2$ are like those for $CaCl_2$ in the case of free osmosis as well as in the case of electrical endosmose.

We have inferred in the preceding paper² that the rate of transport of liquid in electrical endosmose varies, if the other conditions remain equal, with the value of the charge ϵ on the unit area of membrane. If this inference is correct then it follows from the nature of the curves in Fig. 2 that beginning with the lowest concentrations the influence of the anion on the density of charge of the membrane increases at first more rapidly with increasing concentration than the depressing effect of the cation upon the density of this charge, while later the reverse occurs. The turning point seems to lie for the solutions mentioned between M/512 and M/256, where it also lies for free osmosis.

II.

When we separate solutions of electrolytes with a hydrogen ion concentration of 10^{-4} N or above from pure water by collodion membranes which have been treated with a protein, the watery phase of the double layer is negatively and the membrane positively charged.^{1,4} In the preceding paper² it was shown that in such a case the charge of the membrane is increased by the cation and diminished by the anion of an electrolyte in solution, both effects increasing with the valency

⁴ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 717.

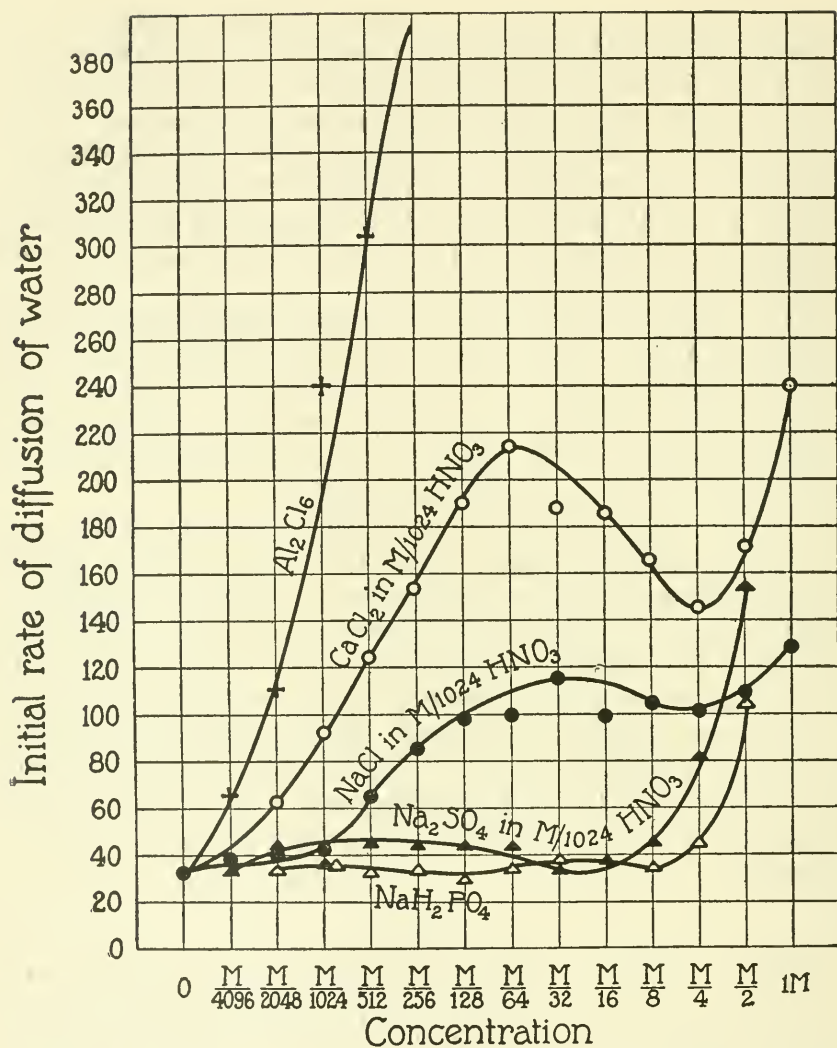


FIG. 3. *Free osmosis.* Effect of ions on free osmosis when water is negatively charged due to acid reaction of solution. Inside the collodion bag acidulated solutions of salts; outside distilled water. Abscissæ are the logarithms of concentration of solution, ordinates rise of level of liquid in manometer after 20 minutes.

of the ion. Fig. 3 represents the influence of different concentrations of electrolytes (with hydrogen ion concentration above 10^{-4} N) upon the rate of diffusion of water through the membrane. It increases with the valency of the cation and diminishes with the valency of the anion, the curves in the case of Na_2SO_4 or NaH_2PO_4 showing no rise. The curves for CaCl_2 and NaCl show a rise and then a drop, the turning point, however, lying at higher concentrations than for neutral solutions. The rise and drop find their explanation on the assumption that the influence of the cation on the charge of the membrane

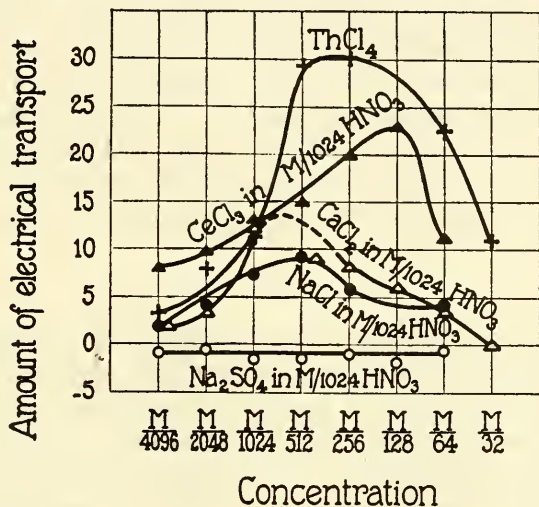


FIG. 4. *Electrical endosmose*. Effect of acidulated salt solutions on electrical endosmose. Inside and outside the bag identical solutions. Similarity of curves of transport in Fig. 4 and Fig. 3.

increases at first more rapidly with increasing concentrations than the depressing effect of the anion upon this charge, while at concentrations above a certain point the reverse happens.

This conception is supported by experiments on electrical endosmose as represented in Fig. 4. The solutions of Na_2SO_4 , NaCl , CaCl_2 , and CeCl_3 were made up in M/1,024 HNO_3 and the hydrogen ion concentration was in the neighborhood of 10^{-3} N. The ThCl_4 solution was sufficiently acid on account of hydrolysis (its pH varying between 3.5 and 1.9 according to concentration). The watery phase was negatively charged and it was necessary to put the anode into the glass

tube in order to bring about a rise in the level of water in the tube. It is obvious from Fig. 4 that the electro-endosmotic transport of the negatively charged liquid rises at first with increasing concentration of the electrolyte and then falls again; and the initial rise increases with increasing valency of the cation and diminishes with increasing valency of the anion. The turning point varies for different electrolytes, probably on account of secondary chemical reactions at the electrodes, especially acid formation at the anode. The curves if interpreted on the basis of Helmholtz's theory prove that when the membrane is positively charged its positive charge is raised in low concentrations of electrolytes more considerably by the cation than it is depressed by the anion of the electrolyte, while when the concentration of the electrolyte exceeds a certain limit the depressing effect of the anion increases more rapidly with further increase in concentration than the opposite effect of the cation; thus supporting the explanation offered for the phenomena of free osmosis in Fig. 3.

III.

Fig. 5 is a repetition of a figure published in a preceding paper⁵ showing the difference of influence of Al_2Cl_6 on the rate of diffusion of water when the collodion membrane has been treated with a protein and when it has not been treated. When the membrane has been treated with a protein, water is powerfully attracted by a solution of Al_2Cl_6 and the attraction increases with the concentration (upper curve); when the membrane has not been treated with a protein, water is not attracted by the solution except when the concentration becomes so high that the gas pressure effect of the solution shows itself (lower curve). The explanation offered by the writer was as follows. When we separate Al_2Cl_6 solutions of sufficiently low concentration from pure water by a membrane treated with gelatin, the water diffuses through the membrane in the form of negatively charged particles which are attracted powerfully by the Al ion and repelled weakly by the Cl ion; or, in other words, the Al ion increases the positive charge of the membrane considerably and the Cl ion depresses the same charge less. As a consequence the density of the positive charge of the membrane on the solution side of the membrane must

⁵ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 255.

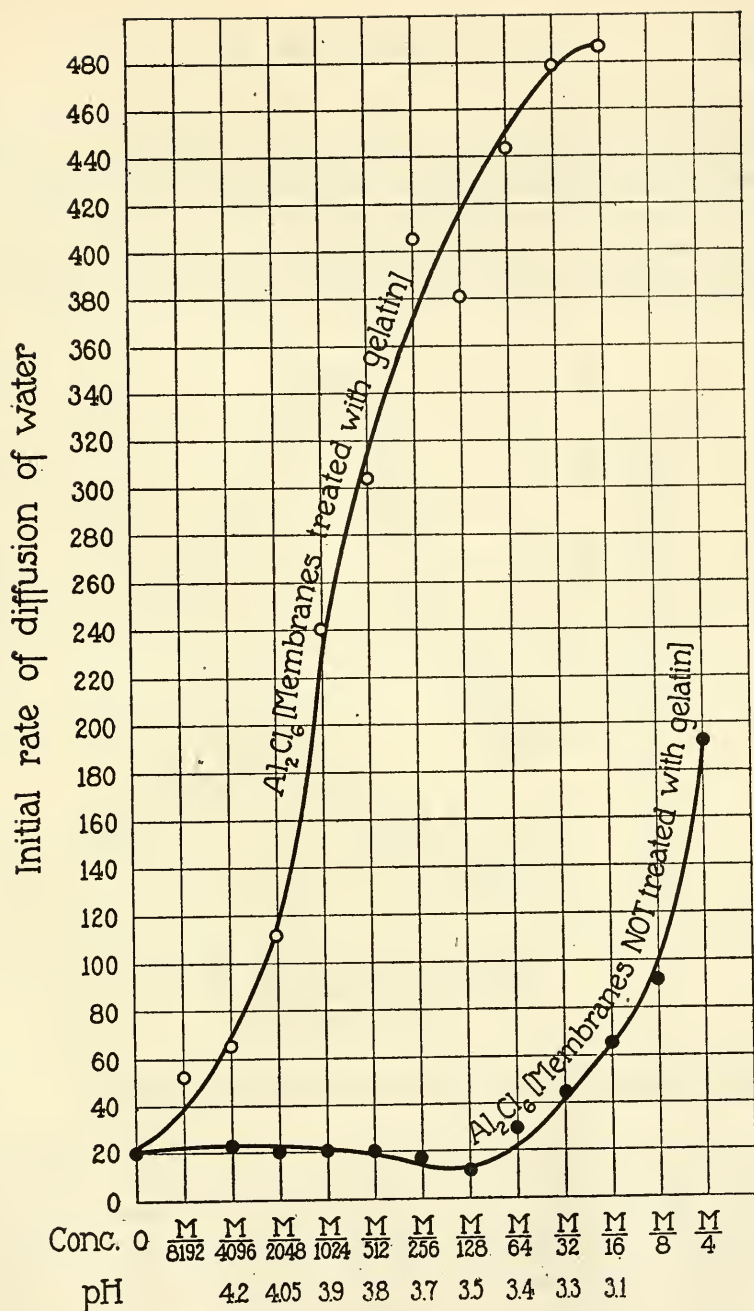


FIG. 5. *Free osmosis.* Upper curve, influence of Al_2Cl_6 upon the rate of diffusion of water when the collodion membrane has been treated with gelatin; lower curve, influence of Al_2Cl_6 on rate of diffusion when the membrane has not been treated with gelatin.

be greater than the positive charge of the membrane on the side of pure water and water will be driven to the solution side. When, however, the collodion membrane has not been treated with gelatin it is negatively charged even in the presence of Al_2Cl_6 and of acid, and in the presence of the Al ions the Cl ions cannot raise the negative charge on the solution side of the membrane beyond that on the water

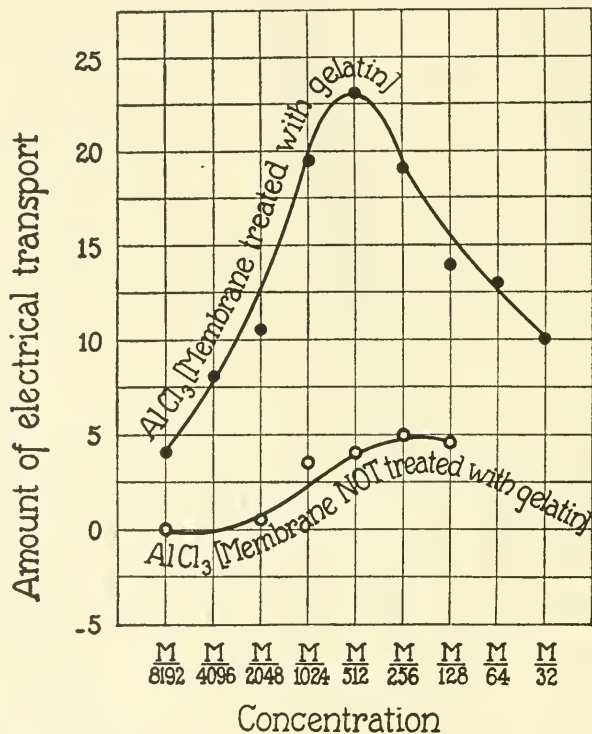


FIG. 6. *Electrical endosmose* with the same two kinds of membrane.

side. As a consequence in this case no other attraction of water by the solution except that based on the gas pressure effect can be produced. If this view is correct, the curves of electro-endosmotic transport of liquid through membranes treated and not treated with gelatin must be similar to the curves of free osmosis in Fig. 5. Fig. 6 shows that this is the case. When identical solutions of AlCl_3 are put into the collodion flask and into the beaker into which the collodion flask is dipped, and when a P.D. is applied in the way described,

a transport of water to the anode occurs which increases with increasing concentration to about $M/512$ provided the membrane had been treated with a protein (upper curve, Fig. 6); when the collodion membrane has not been treated with a protein no such migration occurs (lower curve, Fig. 6). Hence the curves for electro-endosmotic transport of liquid through collodion membranes and the transport of water in free osmosis run parallel, supporting the explanation offered.

In comparing the upper curves in Figs. 5 and 6, the reader will notice a difference, inasmuch as in the case of free osmosis through membranes treated with gelatin (upper curve, Fig. 5) the curve shows only a rise but no drop, while in the upper curve for electro-endosmotic transport (Fig. 6) through a gelatin-treated membrane in the presence of a solution of $AlCl_3$ there occurs the characteristic drop at a concentration beyond $M/512$. The writer is inclined to attribute the reason for this difference to the fact that in free osmosis the gas pressure effect prevents the drop while in electrical endosmosis this gas pressure effect is excluded (since the solutions on both sides of the membrane are identical).

IV.

When we separate a $M/256$ solution of Na_2SO_4 or Li_2SO_4 by a collodion membrane from pure water, the latter will diffuse into the solution with a certain velocity. When we add small and identical quantities of a salt like KCl to the solution of $M/256$ Na_2SO_4 and to the distilled water, this velocity will be diminished;⁶ owing to the fact that beyond a concentration of $M/256$ Na_2SO_4 the repelling or depressing effect of the cation of the solute increases more rapidly with increasing concentration of the electrolyte than the attracting or accelerating effect of the anion on the rate of diffusion. When we add $MgCl_2$ or $CaCl_2$ instead of KCl the depressing effect is still greater than in the case of KCl , owing to the fact that Mg and Ca , as bivalent ions, have a greater depressing effect than K . Fig. 7 illustrates this statement, showing that the addition of KCl or $MgCl_2$ or $CaCl_2$ to $M/256$ Na_2SO_4 diminishes the rate of diffusion of liquid into the Na_2SO_4 solution and that $MgCl_2$ and $CaCl_2$ act more power-

⁶ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 273.

fully than KCl. The addition of 0.2 cc. of $M/4$ $MgCl_2$ or $CaCl_2$ to 100 cc. of $M/256$ Na_2SO_4 (concentration of $MgCl_2$ or $CaCl_2 = M/2,000$) lowers the rate of diffusion of water into the solution more than 50 per cent, while the effect of the addition of 0.2 cc. of $M/4$ KCl (concentration of KCl = $M/2,000$) lowers the rate of diffusion of water into the solution considerably less.

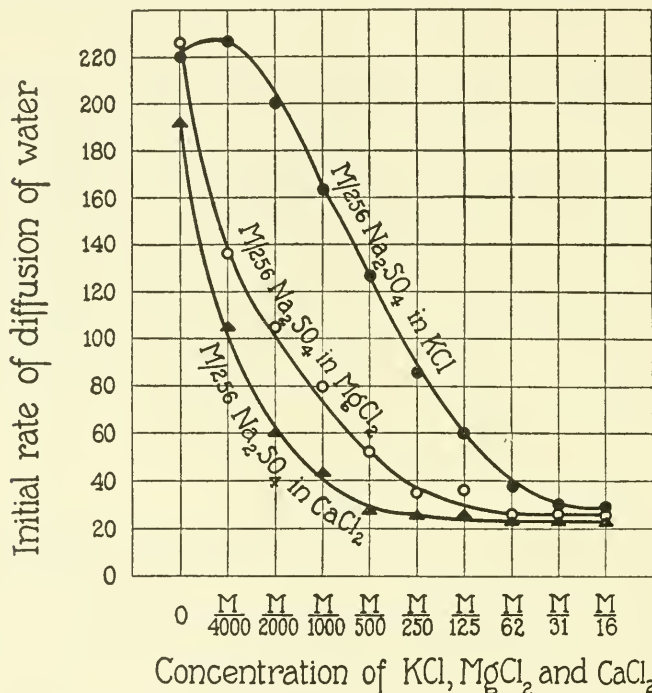


FIG. 7. *Free osmosis*. Depressing effect of the addition of KCl, $MgCl_2$, and $CaCl_2$ upon the attraction of water by $M/256$ Na_2SO_4 .

Fig. 8 shows that the addition of KCl and $CaCl_2$ to $M/512$ Na_2SO_4 has the same depressing effect on the rate of transport of liquid through the collodion membrane in the case of electrical endosmose. In this, as in the preceding experiments on electrical endosmose, identical solutions were put into both sides of the collodion membrane. These solutions were $M/512$ Na_2SO_4 alone or with the addition of small quantities of KCl or $CaCl_2$. A P.D. of 15 volts was produced on the opposite sides of the membrane and the rate of transport of water

(which took place to the cathode) was observed. The curves in Fig. 8 show the result. The quantity of transport of liquid was depressed both by the addition of KCl and of CaCl_2 but more by CaCl_2 than by KCl. The addition of 0.1 cc. of $\text{M}/4$ CaCl_2 to 100 cc. of $\text{M}/512$ Na_2SO_4 (concentration of $\text{CaCl}_2 = \text{M}/4,000$) lowers the transport of water more than twice as much as the addition of 0.1 cc. of $\text{M}/4$ KCl. The depressing influence of the addition of KCl or CaCl_2 in the electrical transport of water through a collodion membrane can be demonstrated equally well with $\text{M}/256$ solutions of Na_2SO_4 as with $\text{M}/512$ solutions.

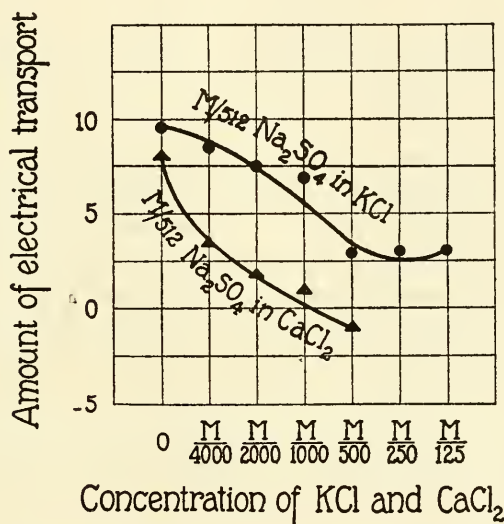


FIG. 8. *Electrical endosmose*. Showing that a similar depressing effect as in Fig. 7 exists in the case of electrical endosmose.

These experiments on electrical endosmose show that the density of negative charge on the membrane in contact with a $\text{M}/256$ or $\text{M}/512$ Na_2SO_4 solution is diminished by the addition of any electrolyte (whose anion does not act more powerfully than SO_4) and that the diminution increases with increase in the valency of the cation added. This furnishes the explanation of the fact that when $\text{M}/256$ Na_2SO_4 is separated from pure water by a collodion membrane the rate of diffusion of the positively charged water into the solution is diminished by the addition of KCl and still more by the addition of MgCl_2 or CaCl_2 .

The writer has duplicated the majority of the experiments he has thus far published on free osmosis by experiments on electrical endosmose and there is a fair degree of similarity in the two cases.

SUMMARY.

1. It had been shown in previous publications that when pure water is separated from a solution of an electrolyte by a collodion membrane the ion with the same sign of charge as the membrane increases and the ion with the opposite sign of charge as the membrane diminishes the rate of diffusion of water into the solution; but that the relative influence of the oppositely charged ions upon the rate of diffusion of water through the membrane is not the same for different concentrations. Beginning with the lowest concentrations of electrolytes the attractive influence of that ion which has the same sign of charge as the collodion membrane upon the oppositely charged water increases more rapidly with increasing concentration of the electrolyte than the repelling effect of the ion possessing the opposite sign of charge as the membrane. When the concentration exceeds a certain critical value the repelling influence of the latter ion upon the water increases more rapidly with a further increase in the concentration of the electrolyte than the attractive influence of the ion having the same sign of charge as the membrane.

2. It is shown in this paper that the influence of the concentration of electrolytes on the rate of transport of water through collodion membranes in electrical endosmose is similar to that in the case of free osmosis.

3. On the basis of the Helmholtz theory of electrical double layers this seems to indicate that the influence of an electrolyte on the rate of diffusion of water through a collodion membrane in the case of free osmosis is due to the fact that the ion possessing the same sign of charge as the membrane increases the density of charge of the latter while the ion with the opposite sign diminishes the density of charge of the membrane. The relative influence of the oppositely charged ions on the density of charge of the membrane is not the same in all concentrations. The influence of the ion with the same sign of charge increases in the lowest concentrations more rapidly with increasing concentration than the influence of the ion with the opposite sign of charge, while for somewhat higher concentrations the reverse is true.

THE REVERSAL OF THE SIGN OF THE CHARGE OF MEMBRANES BY HYDROGEN IONS.

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(Received for publication, March 13, 1920.)

I. INTRODUCTION.

The similarity between the influence of ions on free and on electrical endosmose through collodion membranes leads to the conclusion that this influence is due to an alteration of the density of the electrical charge of the membrane by the ions.¹ This conclusion rests on the assumption that the Helmholtzian theory of the double layer and the formula connecting the volume of transport of liquid with the density of charge is correct. We have seen that on this assumption the observations lead to the conclusion that the ions with the same sign of charge as the membrane increase and the ions with the opposite sign of charge diminish the density of charge of the membrane. Whether, therefore, a given ion increases or diminishes the density of charge of the membrane depends upon the sign of the charge of the membrane, and, hence, the sign of the charge precedes the influence of ions on the density of charge. One of the problems in the theory of the double layer at the boundary of membrane and watery solution is therefore the origin of the sign of the charge of membranes in contact with water.

Chemists consider the double layer at the boundary of membrane and water as ionic in character and as due to the preferential adsorption of one kind of ions by the membrane, while the watery phase of the double layer is formed by the ions with the opposite charge from those adsorbed. This view has been developed with remarkable lucidity and consistency by Perrin² who assumes that the H and OH

¹ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 387, 563.

² Perrin, J., *J. chim. physique*, 1904, ii, 601; 1905, iii, 50; Notice sur les titres et travaux scientifiques de M. Jean Perrin, Paris, 1918.

ions are especially influential in determining the sign of charge of the membrane. In alkaline solutions the OH ions are said to be adsorbed by the membrane, while the mobile, watery stratum of the double layer is formed by the cations; in acid solutions the H ions are thought to be adsorbed while the anions form the mobile stratum of the double layer. When the OH ions are adsorbed by the membrane the latter is negatively charged, when the H ions are adsorbed the membrane is positively charged. This view meets a difficulty in the fact that as a general rule membranes are negatively charged when in contact with neutral water. The adsorption hypothesis meets this difficulty with an additional assumption; namely, that in a neutral solution the OH ions have a greater tendency to be adsorbed by a membrane than the H ions. We should, however, be forced to assume that the preferential adsorption of OH ions occurs also in some cases in acid solutions, since we have seen that collodion membranes (not treated with a protein) are negatively charged even in strong acid solutions.³ Another difficulty was pointed out by Perrin himself, namely that no other monovalent ion except the H and OH ions were able to reverse the sign of charge of a membrane, and he intimated that this might be due to the fact that the velocities of H and OH ions are greater than those of any other ion. But if the velocity determines the relative degree of adsorption of ions then the H ions should be more readily adsorbed by a membrane in neutral solutions than the OH ions.

A second view, which is held chiefly by physicists, considers the formation of an electrical double layer at the boundary of membrane and water as a case of contact electricity, which may be influenced but which need not be caused by ions. Lenard⁴ has shown that when very minute particles are torn off from the free surface of water the minute particles are negatively charged while the water assumes a positive charge. He concludes from this that at the surface of the water there exists an electrical double layer the external stratum of which is negatively charged, while the internal stratum is positively charged. Since he was able to show that such a double layer exists at the surface of water even in a vacuum, this double layer cannot

³ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 255.

⁴ Lenard, P., *Ann. Physik*, 1915, xlvii, 463.

have its origin in an adsorption of ions. He assumes that the double layer at the boundary of water and membrane is also entirely inside the watery phase. This seems, however, to be disproved by an experiment of Coehn and Franken.⁵ A sphere of paraffin dipped into water possesses a high negative charge which can be demonstrated electrometrically when the paraffin is lifted out of the water. If, however, a film of water is caused to adhere temporarily to the surface of the paraffin when it is lifted out of the water, the electrometer indicates no charge as long as this film exists, but indicates a charge the moment the film has disappeared. This leaves no doubt that when a solid is bounded by water the one charge is in the membrane and the opposite charge in the watery phase. The fact that paraffin and solid substances in general are negatively charged when in contact with water is ascribed by Coehn to the difference in the dielectric constant of the two phases. Coehn has found that substances of a higher dielectric constant assume a positive charge when in contact with a substance of a lower dielectric constant. This would explain why membranes in general assume a negative charge when in contact with water since the dielectric constant of the latter is relatively high.

The formation of a double layer at the boundary of two phases is thus, according to Coehn, a phenomenon of contact (or frictional) electricity. Lenard and more recently Frenkel⁶ have offered suggestions concerning the origin of the double layer which make it dependent on the Rutherford model of the atom. Lenard points out that the atoms at the surface of a body are generally oriented in such a way that the electrons are at the surface and the more massive part (the positive nucleus) is towards the interior. This idea has been elaborated by Frenkel⁶ into a theory of surface electric double layers of solid and liquid bodies. According to this theory "double layers must exist on the surface of all liquid and amorphous solid bodies, whatever their chemical constitution. The latter will determine but the magnitude and distribution of electric charges on both sides of the surface."

⁵ Coehn, A., and Franken, J., *Ann. Physik*, 1915-16, xlviii, 1005.

⁶ Frenkel, J., *Phil. Mag.*, 1917, xxxiii, 297.

On the basis of this theory it seems natural that membranes should as a rule assume a negative charge when in contact with water. The fact which requires a further explanation is the possibility of a reversal of this sign of charge.

Perrin has shown that in certain cases acids are able to cause a membrane in contact with water to be positively charged. The fact that addition of alkali to an acid solution restores the original negative charge to such a membrane was explained by Perrin on the assumption that in an alkaline solution the OH ions are adsorbed. This latter assumption seems unnecessary since the addition of alkali to an acid may merely serve to lower the hydrogen ion concentration below the level required to make the membrane positive. It is, therefore, only necessary to explain why hydrogen ions in sufficient concentration impress a positive charge on so many membranes and the following is a contribution to the solution of this problem.

We have seen in the preceding papers of this series that a collodion membrane not treated with a protein always assumes a negative charge when in contact with a watery solution, even if this solution is acid. This was demonstrated in two ways, first by electrical endosmose,¹ and, second, by common osmosis.³ The experiments with electrical endosmose do not lend themselves so well to the exact determination of that hydrogen ion concentration at which the membrane becomes positive on account of electrolysis and the secondary chemical reactions at the electrodes. The method of common osmosis is free from this source of error. When we separate a watery solution from pure water by a collodion membrane the pure water diffuses into the solution at a rate which is not only a function of the gas pressure of the solute but also of the electrostatic forces of the ions in solution. The rate increases with increasing valency of the anion and diminishes with increasing valency of the cation. When, however, the collodion membrane has been treated for some time with a protein it assumes a positive charge when the hydrogen ion concentration exceeds a certain value; and in this case the watery phase of the double layer assumes a negative charge. This is proved by the fact that when we separate a watery solution with a sufficiently high hydrogen ion concentration from pure water by a collodion membrane

treated with a protein, the water will diffuse into the solution at a rate increasing with increasing valency of the cation and diminishing with increasing valency of the anion. It is not difficult to find out by this method the critical hydrogen ion concentration at which the reversal in the sign of charge of the membrane occurs. The experiments to be described in this paper have led to the result that this critical hydrogen ion concentration is connected but not identical with the isoelectric point of the protein with which the collodion membrane has been treated.

Proteins are amphoteric electrolytes which can form salts with metals (metal proteinates, *e.g.* Na proteinate) as well as with the anions of acids (protein-acid salts, *e.g.* protein chloride). Whether they do the one or the other depends upon the hydrogen ion concentration of the solution. Below a certain hydrogen ion concentration the proteins form salts of the type of metal proteinates, *e.g.* Na gelatinate; above this critical hydrogen ion concentration they form salts of the type of protein-acid salts; *e.g.*, gelatin chloride.⁷ Between the two concentrations proteins form salts with neither cation nor anion, and this is the so called isoelectric point. For a number of proteins (gelatin included) the isoelectric point lies in the neighborhood of a hydrogen ion concentration of 2×10^{-5} N; for oxyhemoglobin it lies at a very different hydrogen ion concentration; namely, 1.8×10^{-7} N. We will show in this paper that the hydrogen ion concentration required to produce a positive charge in a collodion membrane previously treated with a protein varies in the same sense as the isoelectric point of the protein used.

II. Membranes Treated with Gelatin.

The isoelectric point of gelatin is, according to Michaelis, at a hydrogen ion concentration of about 2×10^{-5} N (or in Sørensen's logarithmic symbol, pH = 4.7). A 1 per cent solution of gelatin was put into a collodion bag over night and was removed the next morning. The bag was rinsed a number of times (ten or twenty times or more) with water to remove all the gelatin except that film which remained apparently attached to the inner side of the col-

⁷ Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 39, 237, 363, 483, 559.

lodian membrane. When such membranes were used for osmotic experiments it could be shown that the membrane had a positive charge as soon as the hydrogen ion concentration exceeded 10^{-4} N or a value slightly below 10^{-4} . The point of reversal was therefore near the isoelectric point of gelatin but on the acid side of that point.

The reader will remember that it had been shown in the preceding papers that when we separate a solution of an electrolyte by a collodian membrane from distilled water the water will be attracted by that ion which has the opposite sign of charge and will be repelled by the ion which has the same sign of charge as the watery phase of the double layer, and that both the attractive and repulsive effects increase generally with the valency of the ion.¹ In the case of a salt like CaCl_2 the repulsive effect of Ca upon positively charged water prevails over the attractive effect of Cl upon such water and no diffusion of such water into the CaCl_2 solution will occur; while when the water is negatively charged the attractive effect of Ca prevails over the repulsive effect of the Cl ion upon the water and water will diffuse rapidly into the solution. We therefore can use a solution of CaCl_2 to find out at which hydrogen ion concentration the reversal of sign in the charge of the watery phase occurs. When we separate a M/256 solution of CaCl_2 from a solution of water by a collodian membrane, water will commence to diffuse into the solution as soon as the water in contact with the membrane assumes a negative charge; while otherwise practically no such diffusion will occur. In these experiments the collodian flasks described in the previous experiments were used. They were closed with rubber stoppers perforated by a glass tube with a bore of 2 mm. in diameter serving as a manometer. The collodian bags were dipped into beakers filled with water. The M/256 solution of CaCl_2 and the water in the beaker into which the bags were dipped were always given the same hydrogen ion concentration; they were rendered acid by the addition of HNO_3 and alkaline by the addition of KOH. We can in this way ascertain at which hydrogen ion concentration the water will commence to diffuse into the solution of M/256 CaCl_2 and this will give us that hydrogen ion concentration where the reversal of the sign of charge on the membrane occurs. Such a curve is given in Fig. 1. The abscissæ are the hydrogen ion concentrations (expressed in terms of pH), the ordinates the rise of

level of liquid in the glass tube after 20 minutes. The reader will notice that the curve for $M/256 \text{ CaCl}_2$ is low and flat as long as the $\text{pH} > 4.0$ and that a sharp rise in the curve begins at a pH of about 4.0 or a little less. The curve rises steeply with diminishing pH (*i.e.* increasing hydrogen ion concentration) until it reaches a maximum at

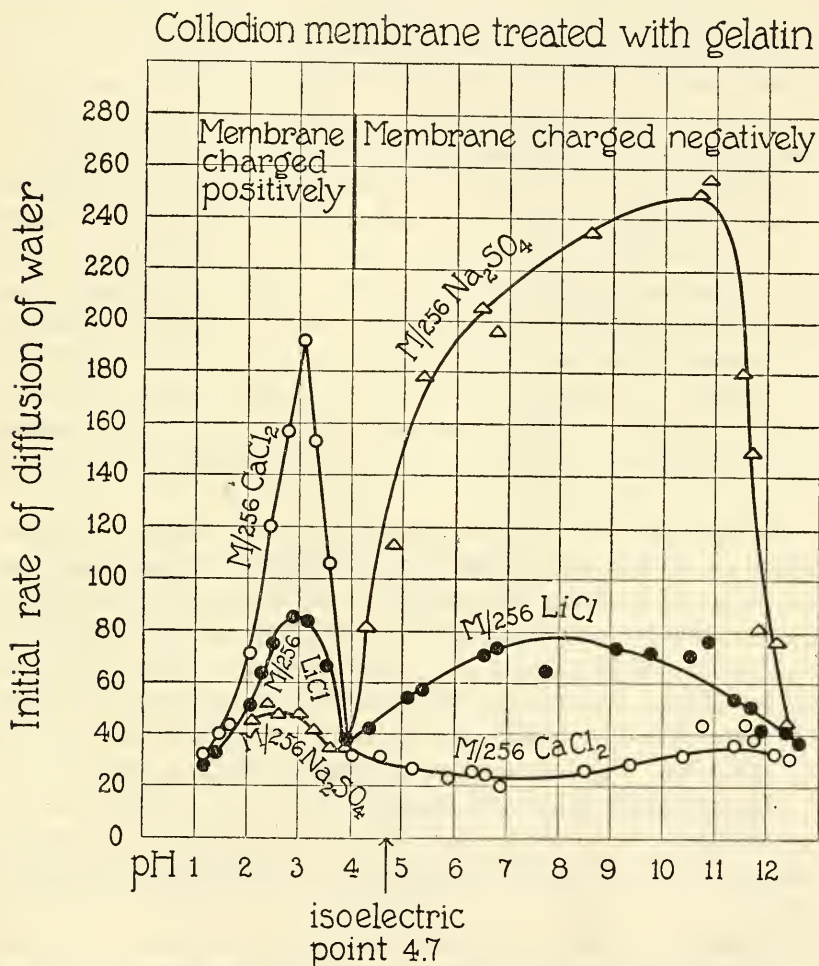


FIG. 1. Collodion membranes previously treated with 1 per cent gelatin solution. Abscissæ, pH (logarithms of hydrogen ion concentration with minus sign omitted). Ordinates, initial rate of diffusion of water from pure water through collodion membranes into solutions of salt indicated. Reversal of sign of charge of membrane at $\text{pH} = 4.0$; *i.e.*, on the acid side of the isoelectric point of gelatin.

about $\text{pH} = 3.1$ and then drops again. This drop is the concentration effect discussed in preceding papers and due to the ions with the opposite sign of charge as that of the membrane, in this case Cl and NO_3 (the latter being added with the nitric acid).¹

Instead of using CaCl_2 we can also use a $\text{M}/256$ solution of Na_2SO_4 as a test for the sign of the electrification of water. We put $\text{M}/256$ Na_2SO_4 into the collodion flask and dip the latter into H_2O . The outside water as well as the $\text{M}/256$ solution of Na_2SO_4 is brought to the same pH by adding HNO_3 (or KOH). The SO_4 ion attracts positively charged water and the Na ion repels it, but the attractive effect of the SO_4 ion is greater than the repelling effect of the Na ion. Water will commence to be attracted by $\text{M}/256$ Na_2SO_4 as soon as the water is positively charged; when water is negatively charged it will be repelled more powerfully by SO_4 than it will be attracted by Na . Hence the hydrogen ion concentration at which the water commences to be positively charged will be indicated by a rise in the level of liquid in the glass tube serving as a manometer. Fig. 1 shows that water commences to be positively charged at a pH of about 4.0 or slightly above where the turning point was also found when we used $\text{M}/256$ CaCl_2 solution as a test.

We can finally use solutions with monovalent anion and monovalent cation as a test solution; *e.g.*, $\text{M}/256$ LiCl . In this case we get an attraction for water both in alkaline and in acid solutions. In the acid solution the negatively charged water is attracted by the Li ion and in the alkaline solution the positively charged water is attracted by the Cl ion. Between the two effects there should be a point where the water is neither positive nor negative and hence is not attracted by either ion. Fig. 1 shows that this point lies again at a pH near 4.0.

Fig. 2 gives the point of reversal in the presence of $\text{M}/256$ solutions of NaH_2PO_4 , Na_2 oxalate, and NaCl . The point of reversal lies between 4.4 and 4.0, but the steep rise of the curve commences at pH 4.0.

Experiments with a number of other salts and other acids were made, all giving the same result; namely, that the membrane commences to be distinctly positively charged as soon as the pH is 4.0 or slightly above, but below 4.7. At the isoelectric point, $\text{pH} = 4.7$, no acid combines with the gelatin and no change of the sign of charge

can be expected at this point. When the hydrogen ion concentration of the solution becomes higher than this, an increasing amount of gelatin is transformed into gelatin-acid salt. With an increase in the concentration of the gelatin-acid salt an increasing proportion of the surface of the membrane assumes a positive charge. Hence the steep

Collodion membrane treated with gelatin

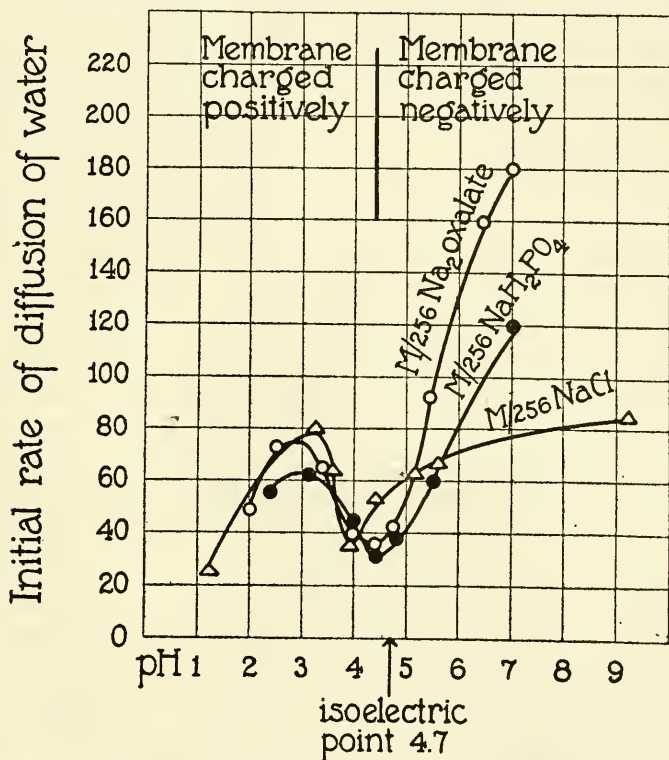


FIG. 2. Collodion membranes treated with gelatin. Reversal of sign of charge of membrane on acid side of the isoelectric point of gelatin.

rise of the curves in which $CaCl_2$ or $MgCl_2$ are used as test solutions must lie near the isoelectric point but on the acid side from the latter.

It may be pointed out that the influence of acids on the reversal of the sign of charge is not an additive effect of the oppositely charged

ions, as was the case in regard to the influence of ions on the density of charge of the membrane.¹ If this were the case, SO_4 should shift the steep rise of the curve more to the acid side and Ca more in the opposite direction. The curves given in this paper (and many other curves not published) show that this is not the case. This supports the view that the mechanism for the influence of ions on the density of charge and for the influence of the hydrogen ions on the reversal of the sign of charge is not the same.

The curves in Fig. 1 show that there are three relative minima of the charge of the membrane. One lies at about $\text{pH} = 4.0$ or slightly above, when the membrane is neither positively nor negatively charged. The drop leading to this minimum is not an additive function of the oppositely charged ions and differs in this respect from the drop leading to the other two minima, the latter being due to the diminution of the density of charge on the membrane caused by that ion which has the opposite sign of charge as the membrane. We have shown in the preceding experiments¹ that this depression occurs when the concentration of the electrolyte exceeds a certain value. One of these minima lies on the acid side, namely at a pH less than 2, owing to the fact that the concentration of the HNO_3 added is $\text{M}/100$ or more. This minimum is due to the depressing effect of the anions (NO_3 and SO_4) of the solution upon the density of the positive charge of the membrane. The third minimum lies at a pH of about 12 or above and is caused by the depressing effect of the cation of the solution, Na, K, etc., upon the density of the negative charge of the membrane. If we had continued to increase the concentration of acid there would have been another rise of the curve due to the gas pressure effect of the acid solution; and the same might have happened if we had been able to increase the concentrations of the KOH added; strong alkali solutions, however, dissolve the membrane.

III. Membranes Treated with Casein and Egg Albumin.

The isoelectric point of casein is, according to Michaelis,⁸ identical with that for gelatin, since it lies at a pH of about 4.7. When we treat collodion membranes over night with a 1 per cent casein solution in-

⁸ Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914.

stead of with gelatin solution, and make the same experiments as described for gelatin, we should expect to find the hydrogen ion concentration at which the collodion membrane treated with casein is rendered positive to be identical with that found for membranes treated

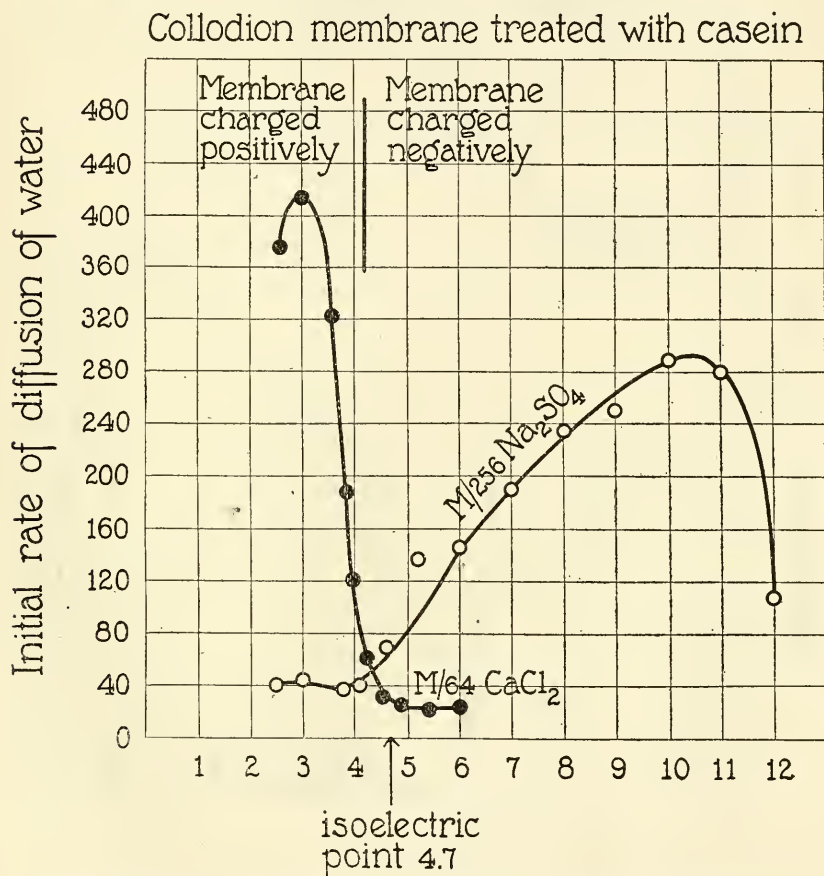


FIG. 3. Collodion membranes treated with casein. Reversal of sign of charge of membrane at pH below 4.7, on the acid side of the isoelectric point of casein.

with gelatin. Fig. 3 shows that this is correct. The curves for membranes treated with casein show a steep rise at a pH of 4.0 or slightly above, but below 4.7. The same is true for membranes treated with egg albumin (Fig. 4). The curves show a steep rise

Collodion membrane treated with egg albumin

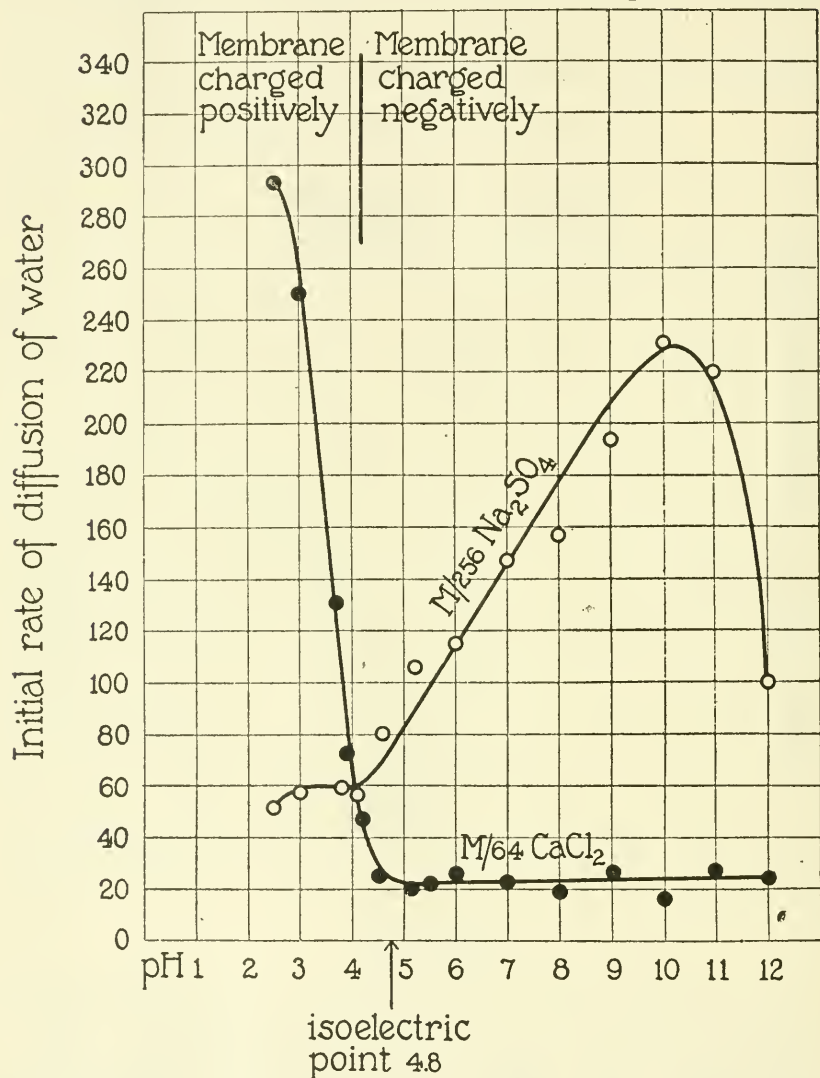


FIG. 4. Collodion membranes treated with egg albumin. Reversal of sign of charge of membrane at pH near 4.0, on the acid side of the isoelectric point of egg albumin.

at a pH of about 4.0. The isoelectric point of egg albumin is, according to Sørensen,⁹ at 1.5×10^{-5} N or pH about 4.8.

IV. Membranes Treated with Oxyhemoglobin.

Defibrinated ox blood was diluted repeatedly with isotonic solutions of NaCl and centrifuged to remove practically all the proteins

Collodion membrane treated with oxyhemoglobin

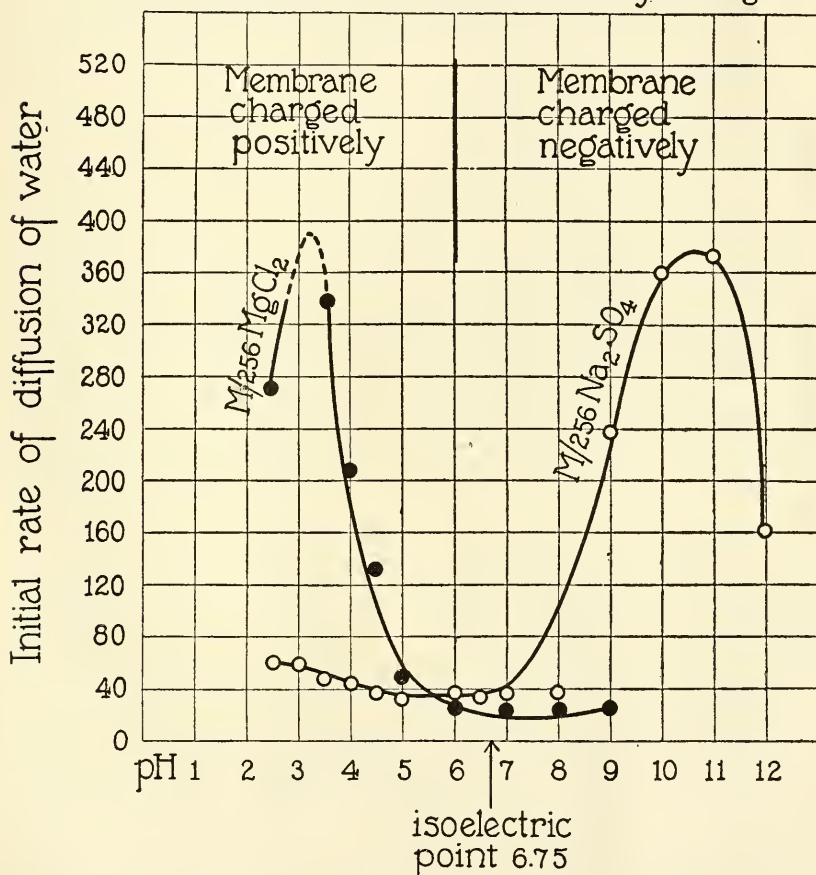


FIG. 5. Collodion membranes treated with oxyhemoglobin. Reversal of sign of charge of membrane at pH of about 6.0, on the acid side of the isoelectric point of oxyhemoglobin.

⁹ Sørensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915-17, xii.

of the blood except those contained in the red corpuscles. After repeating this process five or more times the corpuscles were laked by adding four times their volume of distilled water. Newly prepared

Collodion membrane treated with oxyhemoglobin

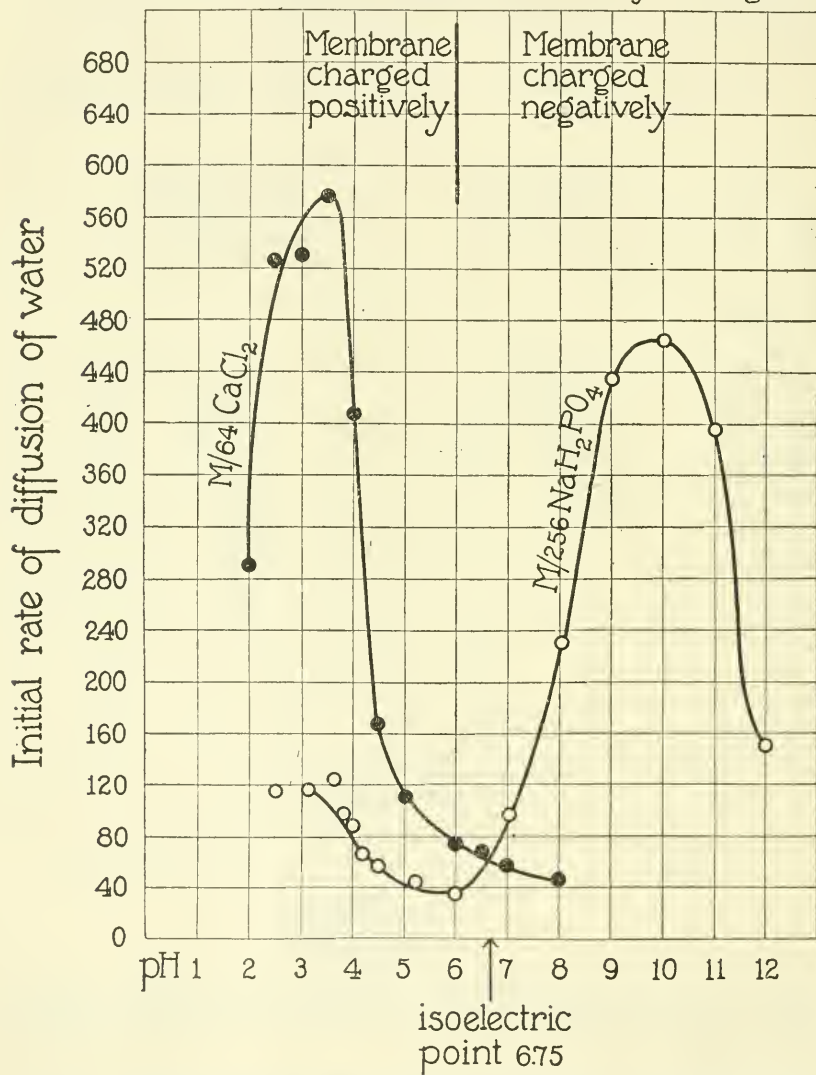


FIG. 6. Collodion membranes treated with oxyhemoglobin. Reversal of sign of charge at pH of about 6.0, on the acid side of the isoelectric point of oxyhemoglobin.

collodion bags were filled over night with this crude oxyhemoglobin solution and the bags were used the next day for similar experiments to those described for membranes treated with gelatin. Fig. 5 shows the results of such experiments. The MgCl_2 curve begins to rise at a pH of 6.0 while the curve for Na_2SO_4 rises at a pH of about 7.0 or 8.0. Hence when the membrane has been treated with oxyhemoglobin a much lower hydrogen ion concentration is required to induce a positive charge on the membrane than when the membrane has been treated with gelatin. This was to be expected if the reversal of the sign of charge of a membrane treated with a protein depends on the isoelectric point of the protein. According to Michaelis the isoelectric point of oxyhemoglobin is $\text{pH} = 6.7$ to 6.8 , and we notice accordingly that the membrane treated with oxyhemoglobin assumes a positive charge when the pH is a little less than 6.7 ; *e.g.*, 6.0 . In Fig. 6 the curve for NaH_2PO_4 shows a distinct rise at a pH of 6.0 and that for CaCl_2 at about 6.0 or a little less. In judging the curves in Figs. 5 and 6 the reader must bear in mind that the CO_2 of the air lowers the pH during the experiments and although the experiments lasted only 20 minutes the error so caused was noticeable in the neighborhood of the point of neutrality. Thus the beginning of the rise in the curve for Na_2SO_4 was in reality not at $\text{pH} \approx 8$, but at a lower pH, either 7.0 or probably less.

V. Collodion Membranes not Treated with a Protein.

When we use membranes *not* treated with gelatin and repeat the experiments described in Fig. 1, we get altogether different results (Fig. 7). There is no minimum or drop at pH of about 4.0 or 6.0 since the sign of charge of collodion membranes not treated with proteins is not reversed by acid.³ Collodion membranes not treated with a protein are always negatively (and the watery phase positively) charged within the range of our experiments. Hence a $\text{M}/256$ solution of CaCl_2 cannot attract pure water through such a membrane even in acid solution and the curve for the effect of $\text{M}/256$ CaCl_2 is flat for the whole range of hydrogen ion concentrations (up to 10^{-2} N). The curve for $\text{M}/256$ Na_2SO_4 does not reach a minimum at pH 4.0 but drops slowly reaching a minimum at pH 2.0 or below, this drop being due to the depressing effect of the high concentration of the cat-

ions Na and H of the solution on the negative charge of the membrane. The drop of the curve at the alkaline end (pH = 10 to 12) is due to the high concentration of the cations Na and K, the latter introduced with the KOH added.

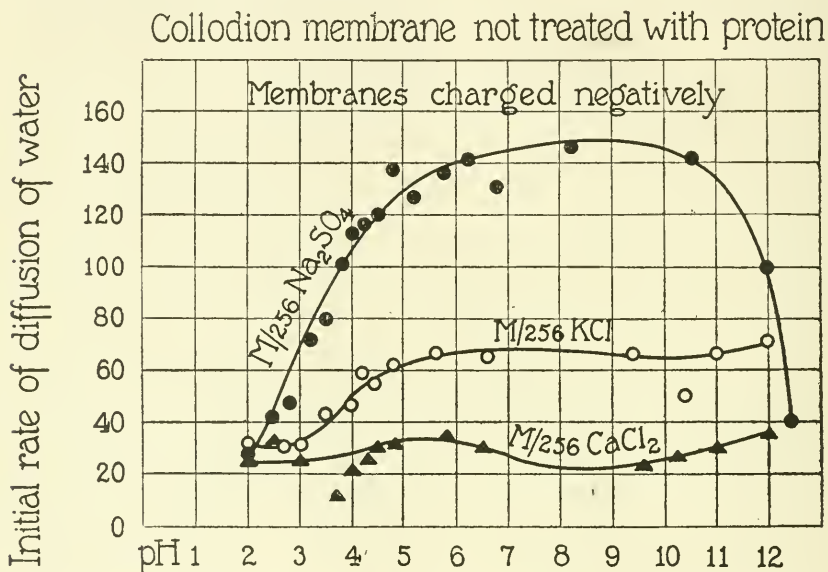


FIG. 7. Collodion membranes not treated with protein. No reversal of sign of charge of membrane.

VI. Theoretical Remarks.

Collodion membranes treated with oxyhemoglobin in the way mentioned remain red after they are washed, showing that the treatment of the membrane with a protein solution results in the formation of a durable protein film at the surface of the collodion membrane and probably also in the interstices of the membrane, since traces of oxyhemoglobin or at least of red pigment diffuse out of the membrane. Applying Werner's idea concerning the combination between acid and NH_3 to proteins we should expect that acids are added to an amino-acid group of a protein in a way similar to that in which they are added to NH_3 ; namely (if the acid added is HNO_3), by forming a salt of the type $R - N \begin{smallmatrix} HNO_3 \\ H_2 \end{smallmatrix}$ which dissociates into a positive $R - NH_3$ ion

and a negative NO_3 ion. On this assumption, the double layer at the boundary of the protein film and a solution would appear to be ionic in character, the positive stratum of the double layer being formed by the protein cations of the surface films of the membrane, while the mobile, watery stratum of the double layer contains the anions of the protein-acid salt. On this assumption it is clear why the hydrogen ion concentration required to make the membrane positive varies in the same sense as the isoelectric point of the protein used and why it is always higher than that of the isoelectric point, since a certain part of the surface of the protein film must be transformed into protein-acid salt before the membrane assumes a positive charge. At a hydrogen ion concentration below that of the isoelectric point the protein film is negatively charged since the protein exists here in the form of metal proteinate dissociating into a protein anion and a metal ion, the latter forming the positive watery stratum of the double layer, while the membrane owes its negative charge to the protein anion.

It does not follow, however, that this transformation of protein into protein-acid salt is the only, or even the essential, condition for the reversal of the sign of charge of the double layer by acid. Since acid can bring about a reversal in the electrical double layer of water bounded by air (or possibly even in a vacuum), it is obvious that the modification of the surface layer of the water which is in contact with the membrane may also play a rôle in the reversal of the sign of charge of the protein film. This possibility has to be considered also in view of the fact that trivalent or tetravalent cations can bring about a reversal in the sign of charge of a membrane treated with protein, even when the hydrogen ion concentration excludes a salt formation between protein and trivalent cation. These facts will be discussed in a subsequent paper.

SUMMARY.

1. It had been shown in previous papers that when a collodion membrane has been treated with a protein the membrane assumes a positive charge when the hydrogen ion concentration of the solution

with which it is in contact exceeds a certain limit. It is pointed out in this paper that by treating the collodion membrane with a protein (*e.g.* oxyhemoglobin) a thin film of protein adheres to the membrane and that the positive charge of the membrane must therefore be localized in this protein film.

2. It is further shown in this paper that the hydrogen ion concentration, at which the reversal in the sign of the charge of a collodion membrane treated with a protein occurs, varies in the same sense as the isoelectric point of the protein, with which the membrane has been treated, and is always slightly higher than that of the isoelectric point of the protein used.

3. The critical hydrogen ion concentration required for the reversal seems to be, therefore, that concentration where enough of the protein lining of the membrane is converted into a protein-acid salt (*e.g.* gelatin nitrate) capable of ionizing into a positive protein ion (*e.g.* gelatin) and the anion of the acid used (*e.g.* NO_3).

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THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

VOLUME II, NO. 6

JULY 20, 1920



PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

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THE INFLUENCE OF THE SUBSTRATE CONCENTRATION
ON THE RATE OF HYDROLYSIS OF PROTEINS
BY PEPSIN.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, May 18, 1920.)

In contrast to the numerous papers on the influence of changes in the pepsin concentration, the influence of varying the protein concentration on the rate of digestion of protein has been but little studied. Weis¹ found that the rate was nearly directly proportional to the protein concentration in low concentration but increased more slowly than the latter in concentrations of more than 2 to 3 per cent. The experiments were made with a crude enzyme preparation which contained several proteolytic enzymes, and were made in such a way as to compare the changes in different solutions after the same time interval, instead of comparing the times required to cause an equal change. They are therefore difficult to interpret.

Preliminary experiments made with a purified pepsin and purified egg albumin showed in general the same results as those found by Weis. In concentrations of more than 2 to 3 per cent the rate of digestion increases more slowly than the protein concentration and finally becomes nearly independent of it. This phenomenon is a very general one in enzyme reactions and many explanations have been offered to account for it. Brown² suggested that the relative decrease in the rate of digestion with increasing substrate concentration was due to the fact that the enzyme remained combined with the substrate for a period of time large compared with the time necessary for combination to take place. The enzyme therefore becomes more and more "saturated" with substrate as the relative concentration of substrate to enzyme increases. Van Slyke and Cullen³

¹ Weis, *Med. Carlsberg Lab.*, 1903, v, 127.

² Brown, A. J., *J. Chem. Soc.*, 1902, lxxxi, 373.

³ Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 141.

showed that an equation might be derived on the assumption of the above mechanism, based on the law of mass action. The validity of this derivation has, however, been questioned by Falk.⁴ Bayliss⁵ has advocated the view that the combination is due to adsorption and cannot be considered a mass action phenomenon. He states, as do Armstrong and Armstrong,⁶ that the fact that the rate of hydrolysis in many cases is nearly independent of the total substrate concentration cannot be explained on the law of mass action, and must be due to some saturation effect of the enzyme with the substrate.

The fact is frequently overlooked that purely chemical catalysis in strictly homogeneous solutions also shows apparent divergences from the mass law. This point is, however, discussed by Bredig,⁷ Mellor,⁸ Lewis,⁹ and especially by Falk.¹⁰ In the hydrolysis of cane sugar by acids, for instance, the rate of hydrolysis is not proportional to the total concentration of acid used but to the hydrogen ion concentration. As is well known, the hydrogen ion concentration in heavily "buffered" solutions is almost independent of the total acid concentration in certain ranges so that the hydrolysis of cane sugar by such a solution would show an analogous behavior to enzyme reactions in that the rate of hydrolysis of the sugar would be nearly independent of the total acid concentration. The apparent discrepancy of the mass law is therefore due to the fact that the "active concentration" (on which the mass law is based) is in many cases not identical with the total concentration. There is a further discrepancy in these cases due to the fact that the rate of hydrolysis in certain concentrations is not proportional to the C_H^+ as determined by the conductivity ratio. This is the so called salt effect and is probably

⁴ Falk, K. G., *J. Biol. Chem.*, 1916-17, xxviii, 389.

⁵ Bayliss, W. M., *The nature of enzyme action*, Monograph on Biochemistry, London, New York, Bombay, and Calcutta, 3rd edition, 1914.

⁶ Armstrong, E. F., and Armstrong, H. F., *Proc. Roy. Soc. London, Series B*, 1913, lxxxvi, 561.

⁷ Bredig, G., *Ergebn. Physiol., 1te Abt.*, 1902, i, 134.

⁸ Mellor, J. W., *Chemical statics and dynamics*, London, 1904.

⁹ Lewis, W. C. McC., *A system of physical chemistry*, London, New York, Bombay, Calcutta, and Madras, 1919, i.

¹⁰ Falk, K. G., (in press).

due to increased activity of the hydrogen ions by the salt. In any case, it is not due to any peculiarity of the catalytic reaction since the same discrepancy is found in comparing the hydrogen ion concentration as determined by the conductivity and E.M.F. methods. The apparent discrepancy between the mass action law and the kinetics of acid catalysis as outlined above is analogous to the case in enzyme reactions where the rate is not proportional to the enzyme concentration.

Acid hydrolysis, moreover, also shows the same peculiarity in regard to the sugar concentration, *i.e.* the rate does not increase directly as the sugar concentration, as expressed in grams or molecules per liter. In the case of acid hydrolysis the rate increases more rapidly than the concentration. Arrhenius¹¹ has suggested that this behavior is due to the fact that the active concentration of sugar is not correctly expressed by the molecular concentration and has shown that very much better results are obtained if the osmotic pressure of the sugar solution is used as a measure of the active concentration. He further assumes that the acid affects the equilibrium between active and inactive sugar molecules and so accounts for the "salt effect." The same mechanism is assumed to account for the effect of temperature, which is much greater than that predicted by the kinetic theory. This hypothesis, of course, fits the facts, but in the absence of independent evidence is really an assumption of the law of mass action rather than a proof of the law. Several authors have proposed explanations for catalytic reactions on the same basis; *i.e.*, that the catalyst changes the concentration of certain molecules and so increases the speed of the reaction. Stieglitz¹² and his coworkers have been able to verify this hypothesis experimentally in the case of the acid hydrolysis of imido esters. This reaction shows the same peculiarities as that found in many enzyme reactions; namely, the rate is not proportional to the total ester concentration. Stieglitz was able to show, however, that the rate was directly proportional to the concentration of ester ions. He considers that the acid causes the formation of imido ester salts and

¹¹ Arrhenius, S., *Z. physik. Chem.*, 1899, xxviii, 317.

¹² Stieglitz, J., and collaborators, *Am. Chem. J.*, 1908, xxxix, 29, 164, 402, 437, 586, 719.

thereby increases the concentration of active ions. He was able to confirm this by independent measurement of the ion concentration (by means of the conductivities). It follows, as emphasized by Stieglitz, that if the above mechanism is correct so called catalytic reactions are merely limiting cases of ordinary reactions in which the combination of the "catalyst" with the substrate or with the products of hydrolysis is too small to be measured. It seems probable that enzyme reactions are of the same type. There is no doubt, at least, that the enzyme often combines with the products of the reaction and so shifts the equilibrium. Bodenstein and Dietz¹³ have shown experimentally that this is true in certain cases. It would seem better, therefore, to consider enzyme reactions as cases of bimolecular reaction in which one of the products dissociates more or less completely with the liberation of active enzyme; if the dissociation is complete the result would be a monomolecular reaction and, if no dissociation whatever takes place, a bimolecular reaction. Most enzyme reactions are apparently intermediate. The specificity of enzyme reaction thus becomes neither more nor less remarkable than the specificity of any other chemical reaction. (The author has had the privilege of discussing the above points with Dr. K. G. Falk who has reached independently similar conclusions.) It was shown in a previous paper¹⁴ that the above conception of enzyme reactions as applied to pepsin gives a quantitative explanation for the kinetics of the reaction and explains the fact that the rate is not always proportional to the total concentration of pepsin. Arrhenius¹⁵ has pointed out that it also gives the explanation of Schütz's rule and the divergence from the monomolecular law.

It is clear from the brief account of catalytic reactions given above that the same apparent divergences from the law of mass action are to be found in these reactions as in enzyme reactions and that the divergences in many cases at least are caused by the fact that the active concentration is not the same as the total concentration of substance. It seems quite probable that the same explanation applies to both. There is no doubt that the saturation theory is sufficient

¹³ Bodenstein and Dietz, *Z. Elektrochem.*, 1906, xii, 605.

¹⁴ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 471.

¹⁵ Arrhenius, S., *Med. Nobelinst.*, 1908, i.

to explain many of the facts but in the absence of direct evidence it can hardly be considered proved.

According to this theory a certain amount of enzyme can act only on a limited amount of substrate; after this quantity is reached any excess of substrate has no effect on the reaction. It is clear that according to this mechanism it is the ratio of the concentration of substrate to that of the enzyme which causes the relative decrease in the rate of digestion of the substrate as the concentration of substrate increases, and not the actual concentration of substrate present in the solution. If the effect, however, is due to the fact that the active concentration of substrate is not directly proportional to the total concentration then the falling off of the rate of reaction with increasing substrate concentration is independent of the ratio of substrate to enzyme and depends only on the actual concentration of substrate.

It occurred to the writer that this question might be tested experimentally by comparing the rate of digestion of different substrate concentrations when hydrolyzed with different enzyme concentrations. Assume, for instance, that the substrate at concentration $10 S$ is found to hydrolyze five times as rapidly as the substrate at concentration S , when enzyme concentration E is used. According to the monomolecular formula the substrate at concentration $10 S$ should digest ten times as rapidly as the substrate at concentration S . The saturation hypothesis would explain this divergence by the assumption that the enzyme becomes saturated with substrate at a concentration of the latter of less than $10 S$. In concentration $10 S$, therefore, much of the substrate takes no part in the reaction and the rate of reaction is less than the expected. It would be predicted further that increasing the substrate concentration from $10 S$ to $20 S$ would have relatively less effect on the rate of reaction than increasing the substrate concentration from S to $2 S$. This is true. It follows also on the saturation hypothesis that increasing the enzyme concentration from E to $10 E$ should have a relatively greater effect on the rate of digestion of substrate $10 S$ than on the rate of digestion of substrate at concentration S ; since it was assumed in accounting for the effect of increasing the substrate concentration that the enzyme (at concentration E) was more saturated with substrate at (substrate) concen-

tration $10 S$ than at (substrate) concentration S . According to the saturation theory, the rate of digestion in concentration $10 S$ is limited only by the concentration of enzyme while the rate at concentration S is limited both by the concentration of enzyme and by the concentration of substrate; hence changing the enzyme concentration should have a greater effect at substrate concentration $10 S$ than at substrate concentration S . The experiments show that this prediction is not fulfilled. The relative increase in the rate of digestion of substrate at concentration S , caused by increasing the concentration of enzyme from E to $10 E$, is identical with the relative increase in rate of digestion of the substrate at concentration $10 S$, caused by the same increase in enzyme concentration.

If, on the other hand, the relative decrease in rate with increase in concentration of substrate is due to an equilibrium in the substrate solution which causes the concentration of active molecules to differ from the total concentration, the rate of hydrolysis of the substrate at concentration $10 S$ should be always five times the rate of digestion at concentration S (in the example just discussed), irrespective of the enzyme concentration. Experiments show that this is actually the case. It is necessary, of course, in making such experiments to be sure that the range covered is such that the enzyme cannot be considered saturated in both substrate concentrations. That is, the range of substrate concentrations must be such as to show nearly direct proportionality between the rate of digestion and the substrate concentration in the lower, but not in the higher concentrations of substrate. It is also necessary to measure the time required to cause a constant change in the substrate and not a constant percentage change or the change made in a given time. The failure to recognize this has led to much confusion in discussion of the kinetics of enzyme reactions (*cf.* Bredig).⁷

This is due to the fact that in most enzyme reactions the products retard the action of the enzyme. It will be clear therefore (irrespective of the mechanism by which this retardation takes place), that comparative results can be obtained only when a constant amount of products is formed. The actual amount of products formed for example by 10 per cent hydrolysis of varying substrate concentrations will be very different. The larger the concentration of substrate the greater the amount of products formed by 10 per cent hydrolysis and the greater

the consequent slowing up of the enzyme due to the inhibiting effect of the products. It is also clear that the retardation will be proportionally greater if a small amount of enzyme is present than if a large amount is present (irrespective of the mechanism by which the retardation is affected). The same reasoning holds for the case when the amount of products formed in a given time is taken as the measure of the rate of reaction. This question was discussed fully in a previous paper.¹⁴

In all the experiments given in this paper, therefore, the rate of digestion is measured as the reciprocal of the time necessary to cause a small absolute change in the substrate concentration. According to the law of mass action as applied to monomolecular reactions the time necessary to cause this change should be nearly inversely proportional to the substrate concentration, provided the change is small compared to the total change in the lowest concentration. If wider variations than this are used it is necessary to calculate the predicted time according to the monomolecular formula. It may appear that the above method of testing the reaction is a very indirect one and that a simpler and more exact method would be to express the course of a single reaction, according to the mechanism proposed, in a single equation. This equation could then be tested experimentally. Such a procedure, however, leads inevitably to an equation with two or more constants, the value of which must be determined from the experiments themselves, so that but little weight can be attached to the agreement of such an equation with the experimental facts. It seems better, therefore, to limit the experimental conditions in such a way as to leave but one variable.

In all the experiments reported in this paper, the changes are within the above limits and the time required to cause a constant change should, therefore, (according to the mass law) be nearly inversely proportional to the substrate concentration at the beginning of the reaction. As will be seen this is not the case if the total concentration of protein is considered as the active concentration but is approximately true if the concentration of ionized protein is considered as the reacting mass.

In these experiments the rate of hydrolysis was followed by means of changes in the conductivity of the solution. It is, therefore, necessary to be sure that the production of the same amount of peptone in each of the solutions used causes the same change in conductivity. This was tested experimentally in each experiment by adding 1 cc. of peptone solution (prepared from egg albumin by the action of pepsin) to 25 cc. of the protein solution and determining the change in conductivity. It was found that the addition of an equal amount of peptone to protein solutions of varying concentrations (from 20 to 1 per cent) does cause an equal change in conductivity provided the hydrogen ion concentration of the solution is greater than pH 1.8. If the solution is less acid than this the change in conductivity of the solution on the addition of a constant quantity of peptone in the presence of a large amount of protein is less than that caused in the presence of a small amount of protein. This is obviously due to the buffer action of the protein in high concentration and can be foreseen from the titration curve of the protein.

Fig. 1 gives the results of two experiments on the effect of the pepsin concentration on the relative rate of digestion of protein solutions of different concentration. In Experiment 1, (Curve I, Fig. 1) 25 cc. of protein solution containing 8, 4, 2, 1, and 0.5 per cent protein, were hydrolyzed at 25° with the addition of (a) 1 cc. of 2 per cent pepsin, and (b) 1 cc. of 0.2 per cent pepsin. All solutions were brought to a pH of 1.8 with hydrochloric acid. The time necessary to cause a given change (about 1.4×10^{-4} reciprocal ohms) in the specific conductivity was determined.¹⁶ The reciprocal of this

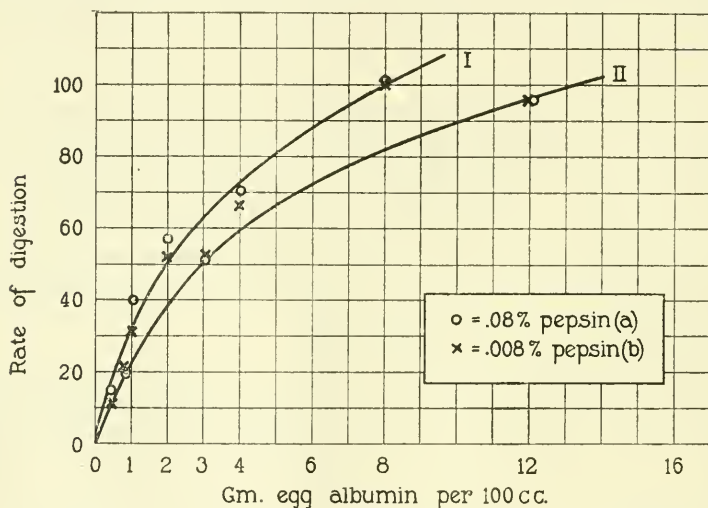


FIG. 1. Relative rate of digestion of egg albumin solutions of different concentration when digested with pepsin solutions of different concentration.

time, therefore, gives the mean rate of digestion of the various solutions for the first 1.4×10^{-4} reciprocal ohm change. In order to compare the two series, the rate of digestion of the concentrated egg albumin (in each series) was considered as 100 and the rate of digestion of the other concentrations calculated on this basis. The curve shows that the relative rate of digestion of the 8 per cent egg albumin compared to the rate of digestion of 4, 2, 1, or 0.5 per cent egg albumin is the same irrespective of whether 2 or 0.2 per cent pepsin was used. The curve also shows that in low concentrations, 0.5 to 2 per cent, the increase in rate is nearly proportional to the increase

¹⁶ Northrop, J. H., *J. Gen. Physiol.*, 1919-20, ii, 113.

in substrate concentration but increases much more slowly in high concentrations. Experiment 2, (Curve II) shows the same result. It was made the same way but at a pH of 1.6. The points in this case are the average of two determinations. They are therefore more reliable and as the figure shows also more nearly equal. In both experiments the relative activities of the two pepsin solutions were as 4.7: 1. It follows from the experiments that this ratio is also inde-

TABLE I.

Influence of Pepsin Concentration on Relative Rate of Digestion of Protein Solutions.

Ratio: $\frac{\text{Rate of hydrolysis of 15 per cent albumin}}{\text{Rate of hydrolysis of 1 per cent albumin}}$ with	
0.08 per cent pepsin.	0.008 per cent pepsin.
8.9	9.1
9.4	9.4
10.0	10.0
9.8	9.7
Average..... 9.52	9.54

Ratio: $\frac{\text{Rate of hydrolysis with 0.08 per cent pepsin}}{\text{Rate of hydrolysis with 0.008 per cent pepsin}}$ in	
15 per cent protein.	1 per cent protein.
5.3	5.2
5.0	5.4
5.0	5.0
4.7	5.6
5.3	5.5
Average..... 5.06	5.34

pendent of the substrate concentration in which the tests were made. (It was shown in a previous paper¹⁴ that the discrepancy in the rate of digestion as compared with the enzyme concentration can be quantitatively explained on the basis of a mass action equilibrium between the pepsin and peptone.) Table I shows a similar experiment in which several duplicate determinations were made at two protein concentrations with two enzyme concentrations. The results are more accurate and also in closer agreement than those shown in Fig. 1.

It seems necessary to conclude from these experiments that the relative decrease in the rate of digestion of protein solutions of increasing concentration is independent (within the limits of error of these experiments) of the enzyme concentration used.

There does not appear to be any direct experimental evidence on the above point in connection with other enzymes. It is frequently stated, however, (Nelson and Vosburgh,¹⁷ Van Slyke and Cullen³) that the velocity of reaction is directly proportional to the enzyme concentration under all conditions and irrespective of the substrate concentration. If this is true it follows necessarily that the relative rate of digestion of various substrate concentrations, when hydrolyzed with any given enzyme concentration, is independent of the enzyme concentration used.

It appears to the writer that this is contrary to the result predicted by the saturation theory. According to this theory it would be predicted that the falling off in the increase in the rate of digestion as compared to the increase in concentration of a protein solution (above a certain low concentration) is due to the fact that at this concentration the enzyme begins to become saturated with substrate; *i.e.*, the time necessary for the enzyme to combine with the substrate becomes small compared with the time during which it remains combined. If this saturation effect becomes noticeable at a concentration of protein of 2 per cent with 0.08 per cent pepsin it should become noticeable at a lower protein concentration with 0.008 per cent pepsin. The experiment shows this is not the case. If anything, the figures show that the rate of digestion of the substrate falls off more rapidly (as compared to the concentration) with the higher pepsin concentration than with the lower. In Table I, which is more reliable owing to the larger number of determinations there is less than 1 per cent difference.¹⁸

It seems necessary to conclude therefore that the relative decrease in the rate of digestion as compared with the increase in protein con-

¹⁷ Nelson, J. M., and Vosburgh, W. C., *J. Am. Chem. Soc.*, 1917, xxxix, 790.

¹⁸ It probably cannot be assumed that, according to the saturation theory, the rate of digestion (caused by increasing the enzyme concentration ten times) should be increased ten times as much in the concentrated as in the dilute substrate concentration. The increase should be large enough to detect, however.

centration is due to some equilibrium in the protein solution itself and is independent of the enzyme concentration.

It is well known that in acid solution protein exists in an ionized condition. The concentration of ionized protein is not directly proportional to the total concentration but will increase more slowly than the total concentration. It is obvious, therefore, that the rate of digestion will be more nearly proportional to the concentration of ionized protein than to the total concentration of protein. The hypothesis, then, that the ionized protein is the form which takes part in the reaction, will allow a nearer approach to the predicted rate of reaction. Pauli¹⁹ has suggested that the enzyme attacks the ionized protein; there seems, however, to be no direct evidence for this view. It can be tested experimentally by comparing the rate of digestion with the degree of ionization of the protein.

The concentration of ionized protein can be determined approximately from the pH and conductivity measurements. If the total conductivity and the hydrogen ion concentration of a solution are accurately known, the conductivity due to the protein-salt ions can be determined by subtracting the conductivity of the free HCl from that of the solution. The validity of this method rests on three conditions: (1) the conductivity of the free HCl in the solution is the same as that of the same concentration of acid in water solution; (2) the C_{Cl^-} is equal to or greater than the C_H^+ ; and (3) the hydrogen ion concentration as determined by the E.M.F. method must agree with that found by the conductivity method.²⁰ The first assumption cannot be tested directly but it has been shown by Hardy²¹ and by Loeb²² that the viscosity of the solution has no significant effect on the conductivity since the viscosity may increase till the solution is nearly solid without an appreciable change in the conductivity. This experiment was repeated and confirmed. The second condition can be shown to hold also by direct measurements of the chlorine ion concentration by means of concentration cells as was done by Manabe and Matula.²³ Many measurements of this kind were made and confirmed those of the above mentioned authors; namely, the chlorine ion concentration is always equal to or greater than the hydrogen ion concentration. It was

¹⁹ Pauli, W., *Arch. ges. Physiol.*, 1910, cxxxvi, 483.

²⁰ For the purpose of these experiments it is only necessary that the conductivity and E. M. F. methods should agree. The absolute value for the C_H^+ is immaterial.

²¹ Hardy, W. B., *J. Physiol.*, 1905, xxxiii, 251.

²² Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 559.

²³ Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

found, however, that if the conductivity of the H^+ and the total Cl^- were subtracted from that of the solution the remaining values were within the limits of error of the measurements. In other words, the conductivity due to the protein ion itself is very small compared to that due to the excess chlorine ion (by excess Cl^- is meant the difference between the total C_{Cl^-} and the $C_{Cl^-} = C_H^+$). Since, however, the protein ion must equal in concentration the excess chlorine ion the value for the conductivity obtained by subtracting the conductivity of the free HCl from that of the solution may be considered as proportional to the amount of ionized protein. (Recent work, by Noyes, Milner, and others, has rendered questionable the exact interpretation to be put upon conductivity ratios; they very probably do not represent the actual ion concentration in all cases.) The third condition may be experimentally fulfilled by standardizing the apparatus used for the C_H^+ determinations against HCl solutions of known conductivity and taking the C_H^+ as that determined by the conductivity ratios. This method was used in the present experiments. The final values for the conductivity due to the protein-salt ions are the difference between two large figures so that the error is very large and becomes larger as the solution becomes more dilute. Below 1 per cent protein solution (at pH 1.7) the value is meaningless as it usually lies within the limit of error.

The egg albumin was crystallized three times and then dialyzed under pressure at the isoelectric point until the specific conductivity was lower than 1×10^{-4} reciprocal ohm. The solutions were then brought to a pH of 1.6 to 1.8 with HCl and then diluted with HCl of exactly the same pH. The solutions varied from 16 to 1 per cent egg albumin. The time necessary to cause a constant small change in the conductivity of the resulting solution by the same amount of pepsin was then determined as described previously.¹⁶ The reciprocal of this time is plotted in the curves as the rate. The conductivity of the solution was measured on an aliquot part of the solution to which the equivalent amount of inactivated pepsin had been added. The C_H^+ was determined by the E.M.F. method on this solution. The value given for the specific conductivity of the protein is obtained by subtracting the specific conductivity of the free HCl from that of the solution. The experimental error of the value is 5 to 10 per cent in the high concentrations and 20 to 30 per cent in the lower. The figures given are the averages of three determinations. All measurements were made at $25^\circ \pm 0.01$.

The conductivity and rate of digestion of the egg albumin was measured in this way. It was found in general that the conductivity of the protein solution was, within the rather large limits of error, directly proportional to the rate of digestion of the solution. In other words, the rate of digestion is that predicted by the mass law if the ionized protein is considered as the reacting form. The results of three such experiments are given graphically in Fig. 2 in which

the rate of digestion is plotted against the conductivity of the protein. This figure shows that the two values are approximately directly proportional.

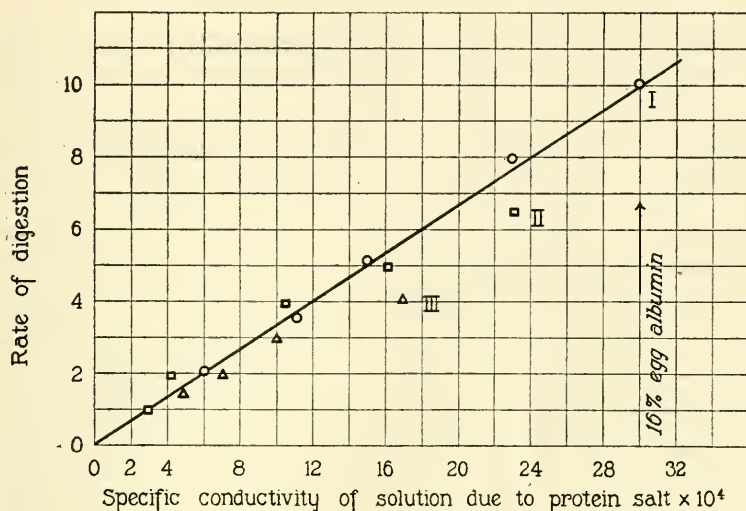


FIG. 2. Rate of digestion and conductivity of egg albumin solutions.

Viscosity of the Solution.

It is impossible to use egg albumin in more concentrated solutions than 16 per cent as the higher concentrations set to a jelly very rapidly. It seemed quite possible that the increasing viscosity of the solution might affect the rate of digestion (as found by Colin and Chaudun²⁴ for invertase). This question can be tested experimentally by taking advantage of the well known hysteresis of albumin solutions.

500 cc. of a 25 per cent egg albumin solution were titrated to pH 1.6 with HCl, placed at 25°, and the viscosity and rate of digestion of a sample determined at intervals for about 10 hours. The amount of pepsin used was such that the viscosity of the digesting solution did not change appreciably during the determination. This was due to the fact that the decrease in viscosity by the pepsin was equalized by the increase of the viscosity with time. The viscosity of the solution at the beginning of the experiment was about three times that of water and at the end too large to measure by the viscosimeter. At the beginning of the last

²⁴ Colin, H., and Chaudun, A., *Compt. rend. Acad.*, 1919, clxix, 849.

digestion test the solution could hardly be pipetted with a wide-mouth pipette and was semisolid. The figures for viscosity are doubtless all too low since the viscosimeter was not known to obey Poiseuille's²⁵ law and almost certainly did not obey it since the time of outflow for 20 cc. of water was only 10 seconds.

The result of the experiment is shown graphically in Fig. 3. The rate of digestion is not affected appreciably, until the viscosity has increased four to five times that of water. This is far greater than the viscosity of any solution used in the other experiments referred to. There is no doubt, however, that when the solution becomes

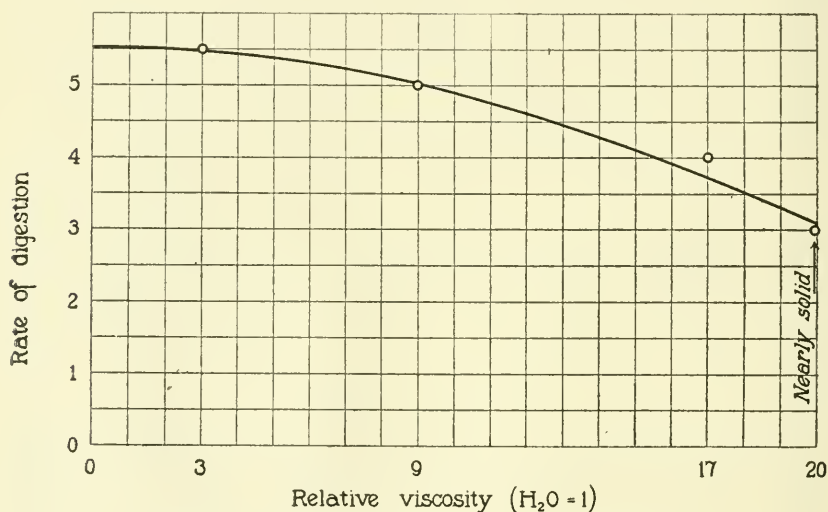


FIG. 3. Influence of viscosity on rate of digestion.

nearly solid the rate of digestion is greatly decreased. It was found that the same decrease was noticed if the viscosity of the solution was increased by the addition of agar. The presence of the agar alone is not the cause of the decrease in the rate as was shown by the fact that the rate of digestion was unaffected by the agar if the experiment was conducted at 40° (when the agar caused no increase in viscosity). It would seem, therefore, that the effect of viscosity must be a mechanical one due to interference with the diffusion of

²⁵ For a discussion of this question see Washburn, E. W., and Williams, G. Y., *J. Am. Chem. Soc.*, 1913, xxxv, 737.

the enzyme rather than to a change in the resistance of the protein. This is borne out by the fact that pepsin diffuses only very slowly through coagulated protein (*cf.* for instance Dauwe).²⁶ Reformatsky²⁷ has shown that the rate of hydrolysis of methyl acetate by acids is identical in water solution and in a solid agar gel. In this case the rate of diffusion of the H^+ is also independent of the viscosity (Voigtländer).²⁸

Ringer²⁹ has pointed out that the optimum pH for the digestion of protein coincides approximately with the maximum viscosity and has suggested that the rate of digestion is dependent on the degree of hydration of the protein; the viscosity of the solution is also assumed to be a measure of the degree of hydration. It would seem from the experiment just described that an increase in viscosity decreases the rate of digestion instead of increasing it, as supposed by Ringer. If the protein ion is the active form of the protein the optimum pH should depend on the maximum degree of ionization. According to Pauli¹⁹ the maximum viscosity also depends on the ionization. Loeb²² has shown, however, that this is not true. The hypothesis outlined above requires that the rate of digestion of a protein solution at different pH should be directly proportional to the amount of protein ionized. Preliminary experiments show that this is true, qualitatively at least. Unfortunately the change in conductivity (as pointed out above) cannot be used to follow the rate of digestion at lower C_H^+ so that the experimental difficulties are much greater.

The results of the present paper may be considered in qualitative agreement at least with the mechanism of pepsin digestion as outlined in the preceding paper.¹⁴ The hypothesis advanced considers that there is an equilibrium in the pepsin solution between pepsin and peptone (substances combining with pepsin and so rendering it inactive). There is also an equilibrium between ionized and unionized protein in the protein solution. The reaction takes place according

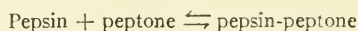
²⁶ Dauwe, F., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 426.

²⁷ Reformatsky, S., *Z. physik. Chem.*, 1891, vii, 34.

²⁸ Voigtländer, F., *Z. physik. Chem.*, 1889, iii, 316.

²⁹ Ringer, W. E., *Arch. Neerl. Phys.*, 1918, ii, 571; *Z. physiol. Chem.*, 1915, xcv, 195. Ringer considers that the charge on the protein is also of importance. This agrees with the present experiments.

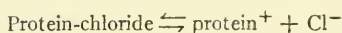
to the law of mass action between the uncombined pepsin and the protein ion. The mechanism may be formulated as below.



or

$$C_{\text{pepsin}} = K \frac{C_{\text{pepsin-peptone}}}{C_{\text{peptone}}} \quad (1)^{30}$$

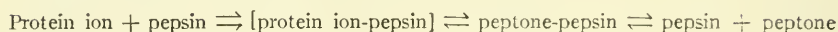
and



or

$$C_{\text{protein ion}} = K \frac{C_{\text{protein-chloride}}}{C_{\text{Cl}^-}} \quad (2)^{30}$$

The reaction would be expressed by



The rate of hydrolysis of the protein at any instant of time would therefore be proportional to the concentration of protein ions and of free pepsin present in the solution at that instant and the differential expression for the rate of reaction would be

$$-\frac{dC_{\text{protein ion}}}{dt} = K C_{\text{protein ion}} \cdot C_{\text{pepsin}}$$

where $C_{\text{protein ion}}$ and C_{pepsin} are determined by equations (1) and (2). There is probably little doubt that the enzyme and substrate unite to form an addition product, but according to the experimental evidence found in this paper the time during which they are combined is negligible in the consideration of the kinetics of the reaction.

The mechanism outlined above will explain, at least qualitatively, the peculiarities in the kinetics of other enzyme reactions. It seems very unlikely, however, that the equilibrium in the substrate solution should always be ionic. It may be any isomeric equilibrium. Since,

³⁰ The equilibrium expressed in (2) is certainly, and that expressed in (1) is probably, influenced by the hydrogen ion concentration.

in such cases, it is extremely difficult to obtain any independent measurement of the equilibrium, there seems to be no way to test the proposed mechanism.

SUMMARY.

1. It is pointed out that the apparent exceptions to the law of mass action found in enzyme reactions may be found in catalytic reactions in strictly homogeneous solutions.

2. These deviations in the rate of reaction from the law of mass action may be explained by the hypothesis that the active mass of the reacting substances is not directly proportional to the total concentration of substance taken.

3. In support of this suggestion it is shown that for any given concentration of pepsin the relative rate of digestion of concentrated and of dilute protein solutions is always the same. If the rate of digestion depended on the saturation of the surface of the enzyme by substrate the relative rate of digestion of concentrated protein solutions should increase more rapidly with the concentration of enzyme than that of dilute solutions. This was found not to be true, even when the enzyme could not be considered saturated in the dilute protein solutions.

4. The rate of digestion and the conductivity of egg albumin solutions of different concentration were found to be approximately proportional at the same pH. This agrees with the hypothesis first expressed by Pauli that the ionized protein is largely or entirely the form which is attacked by the enzyme.

5. The rate of digestion is diminished by a very large increase in the viscosity of the protein solution. This effect is probably a mechanical one due to the retardation of the diffusion of the enzyme.

ENZYME ACTION IN ECHINODONTIUM TINCTORIUM ELLIS AND EVERHART.

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(Received for publication, March 25, 1920.)

In a recent paper¹ it was pointed out that information concerning the physiology of the wood-destroying fungi is comparatively meager. The intention to investigate the enzyme action in some of these forms was also expressed there. The present paper is the second of a series dealing with this phase of metabolism of the wood-destroying fungi.

Echinodontium tinctorium is perhaps one of the most destructive heart rot diseases in the West. White, Alpine, grand, noble, and Douglas fir, Engelmann's spruce, and western and mountain hemlock have been reported as having been affected. Perhaps by far the greatest damage occurs on white fir and western hemlock. The economic importance of this fungus has been sufficiently discussed by Weir and Hubert² and also by Meinecke³ so that no further discussion of its economic importance or distribution is necessary.

The culture of the fungus used in this study was obtained from a young sporophore by the tissue method. The sporophore was carefully washed with sterile distilled water, dried by means of sterile tissue towelling, and cut open. Small portions of tissue were taken from the interior of the fruiting body and transferred to potato agar slants. After the fungus had made considerable growth, transfers were made from the agar slants to sliced sterile carrots in large Erlen-

¹ Schmitz, H., and Zeller, S. M., Studies in the physiology of the fungi IX. Enzyme action in *Armillaria mellea* Vahl., *Daedalea confragosa* (Bolt.) Fr., and *Polyporus lucidus* (Leys.) Fr., *Ann. Missouri Bot. Garden*, 1919, vi, 193-200.

² Weir, J. R., and Hubert, E. E., Forest disease surveys, *U. S. Dept. Agric., Bull.* 658, 1918, 1-23.

³ Meinecke, E. P., Forest trees common in California and Nevada, *U. S. Dept. Agric., Forest Service*, 1914, 1-67.

meyer flasks, and the cultures incubated for 3 months at a temperature of 32°C. The fungus makes comparatively slow growth both on hard potato agar and on the carrots. While still in an actively growing condition the fungous mats were removed from the flasks, and, when thoroughly dry, were finely ground.

All the methods followed in the present study are similar to those in the former paper.¹ This was done in order to make the results as comparable as possible.

Esterases.

The esterase activity of *Echinodontium tinctorium* was determined by the action of the ground fungous meal on methyl acetate, ethyl acetate, ethyl butyrate, triacetin, and olive oil emulsion. After 21 days incubation hydrogen ion concentration determinations of the various enzyme cultures and controls were made and compared. Marked esterase activity occurred when methyl acetate and ethyl acetate were used as substrates, a trace of activity when methyl butyrate was employed, but no apparent activity when triacetin and olive oil emulsion were used as substrates.

Carbohydrases.

Carbohydrase activity is, no doubt, the most important and most interesting phase in the study of the physiology of the wood-destroying fungi with reference to enzyme action. The action of the fungous meal was determined on 1 per cent solutions of maltose, lactose, sucrose, raffinose, potato starch, inulin, white fir, filter paper cellulose, and hemicellulose. After varying periods of incubation the enzyme cultures were filtered and 5 cc. samples of the filtrate treated with 20 cc. of Fehling's solution. In Table I the average results of two titrations are given as the number of cc. of 0.05 N potassium permanganate required to oxidize the dissolved copper oxide. The results indicate evident action on all the substrates.

TABLE I.
The Carbohydase Action of Echinodontium tinctorium.

Incubation period.	Substrate.	With fungous meal.	With fungous meal autoclaved.	Without fungous meal.
		0.05 N KMnO ₄ .		
		cc.	cc.	cc.
14 days	Maltose.....	31.3	19.1	15.1
20 "	Lactose.....	27.5	24.7	21.2
3 hrs.	Sucrose.....	8.2	5.3	0.2
3 days	Raffinose.....	6.1	4.0	0.3
6 hrs.	Potato starch.....	4.4	3.1	0.2
6 days	Inulin.....	7.0	4.5	0.3
30 "	White fir cellulose.....	8.9	3.6	0.2
25 "	Filter paper "	7.1	3.2	0.4
30 "	Hemicellulose.....	8.5	4.8	1.5

Tannase.

Tannase activity was determined by the action of the fungous meal on a 1 per cent solution of tannic acid. After 20 days incubation the cultures were filtered and 5 cc. of the filtrates were titrated against 0.05 N iodine. In all cases negative results were obtained.

Amidase and Urease.

Acetamide and urea were used as substrates to determine the presence or absence of enzymes which split amino-acids into ammonia and hydroxyl acids. The enzyme cultures were set up in wash bottles with the intakes and outlets sealed by means of rubber tubing and clamps. After 10 days incubation the bottles were connected up with other wash bottles containing distilled water and a few drops of bromothymol blue; air was then drawn through the series by means of a suction pump. The change in color due to the shifting of the hydrogen ion concentration and the length of time necessary to cause this change were noted.

When acetamide was used as a substrate, all tests were negative. With urea as a substrate, however, the color of the indicator changed from yellowish brown (pH 5.6) to bright blue in 3 seconds. In the

tests in the controls a slight change in color was also noted, but in no case going beyond the light blue stage even after air had been drawn through the wash bottles for 3 minutes.

Rennet.

The presence of rennet has been variously reported in the wood-destroying fungi. In *Echinodontium tinctorium* fresh milk was coagulated in 2 hours while the controls remained unchanged.

Catalase.

When 50 cc. of a 3 per cent solution of hydrogen peroxide were added to 1 gm. of fungous meal, a rapid evolution of oxygen resulted amounting to 42 cc. of the gas in 1 minute.

Proteases.

Tryptic and ereptic fermentations were studied by the use of 1 per cent solutions of albumin, peptone, casein, and fibrin in enzyme cultures having neutral, acid, and alkaline reactions. In no case was a positive result obtained with either the biuret or the tryptophane test. In order to check the negative results in the fibrin, this material was stained with Congo red and the color fixed by immersing in boiling water. This stained fibrin was then used as a substrate. In such cultures a liberation of the stain would indicate the digestion of the fibrin. In the enzyme cultures having an alkaline reaction, a slight liberation of the stain was noted, but this also occurred in the alkaline controls. Biuret and tryptophane tests with these cultures also were negative.

SUMMARY.

In *Echinodontium tinctorium* the presence of the following enzymes was demonstrated: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, urease, rennet, and catalase.

COMPARATIVE STUDIES ON RESPIRATION.

XI. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE RESPIRATION OF *PENICILLIUM CHRYSOGENUM*.

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(Received for publication, May 28, 1920.)

The effect of hydrogen ion concentration on respiration has received very little attention. Warburg¹ measured the amount of oxygen consumed by fertilized eggs of *Strongylocentrotus lividus*, when they were in a balanced solution of NaCl, KCl, and CaCl₂. He found that on raising the hydrogen ion concentration from pH 8 (which is that of sea water) to pH 6 the oxygen consumption was lowered to nearly one-third, while on lowering the hydrogen ion concentration to pH 11 the oxygen consumption was more than doubled. Loeb and Wasteneys² repeated Warburg's experiments, using *Arbacia punctulata*. They found that if oxygen consumption at an OH ion concentration of 10^{-7} (pH 7) is taken as 1.00, at a concentration of 10^{-4} (pH 10) it was 1.17 and at a concentration of 8.4×10^{-4} (pH 10.92) it was 2.74. Since similar concentrations of NaOH and NH₄OH produced similar effects it appeared doubtful whether the result was to be attributed to the concentration of hydroxyl ions only.

Thunberg,³ studying the effect of H ions on surviving frog muscles, found that with concentrations of 0.005 M HCl (about pH 2.3) the production of CO₂ decreased to 82.7 per cent of the normal, 0.02 M HCl (about pH 1.7) decreased it to 44.6 per cent while with 0.05 M HCl (about pH 1.2) there was a decrease to 24 per cent of the normal. He found the same concentrations of NaOH less toxic; 0.005

¹ Warburg, O., *Z. physiol. Chem.*, 1910, lxi, 305.

² Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxvii, 410.

³ Thunberg, T., *Skand. Arch. Physiol.*, 1910, xxiii, 154; 1911, xxiv, 23.

M (about pH 11.7) decreased the production of CO_2 to 88.8 per cent, 0.02 M (about pH 12.3) decreased it to 71.3 per cent. He found $\text{Ca}(\text{OH})_2$ more toxic than HCl at equal concentrations. He explains this by supposing that calcium precipitates out phosphates in the tissue. Oxygen consumption was decreased to about the same degree that the production of CO_2 was decreased. Thunberg also used a large number of organic acids and found that most of them had only a slight effect in concentrations from 0.01 to 0.2 M. A few, like formic, α -oxybutyric, and pyroracemic acids, decreased the production of CO_2 from 40 per cent to 50 per cent at the greatest concentration. On the other hand acids like citric, fumaric, and malic increased the production of CO_2 in proportion to the increase in concentration of acid.

Some work has been done on the effect of H ions on the fermentation by yeast. In most cases, however, the pH of the solutions was not measured. Solutions of 0.1 N HCl and HNO_3 seem to inhibit fermentation entirely.⁴ Hägglund⁵ determined the pH of his solutions and found that the optimum hydrogen ion concentration for yeast fermentation (using lactic acid, HCl, and H_2SO_4) was 7×10^{-4} (pH 3.16). His criterion of fermentation was the production of CO_2 . Lüers⁶ found that the pH of the solution, after fermentation had been in progress for several days, always was from 2.51 to 2.73, though at the beginning it may have been neutral.

The extent to which oxidase enzymes are concerned in respiration is at present unknown, but it is important to note that they are inhibited by acids. Bertrand,⁷ as well as Abderhalden and Guggenheim,⁸ and later Wolff⁹ reported inhibition of oxidase reaction by acids. According to Bunzell¹⁰ the oxidation of hydrochinone by laccase (from alfalfa) increases with decrease of H ions from 5.6×10^{-8} to 7×10^{-8} (pH 7.26 to pH 8.16). Reed¹¹ found that the

⁴ Drabble, E., and Scott, D. G., *Biochem. J.*, 1907, ii, 340.

⁵ Hägglund, E., *Biochem. Z.*, 1915, lxix, 190.

⁶ Lüers, H., *Z. ges. Brauwesen.*, 1914, xxxvii, 79.

⁷ Bertrand, G., *Compt. rend. Acad.*, 1907, cxlv, 340.

⁸ Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.*, 1907-08, liv, 331.

⁹ Wolff, J., *Compt. rend. Acad.*, 1909, cxlviii, 500.

¹⁰ Bunzell, H. H., *J. Biol. Chem.*, 1915, xx, 697.

¹¹ Reed, G. B., *J. Biol. Chem.*, 1916, xxvii, 299.

oxidase in potato and apple juice was inhibited, when the solution had had hydrogen ion concentrations of 5.5×10^{-4} (pH 3.26) and 7.4×10^{-4} (pH 3.15) respectively, and that in both cases the optimum is near neutrality. Bunzell¹² found that potato juice oxidase was inhibited by pH 3.55 to pH 3.70, and that the activity increased as the pH number increased up to 7. The oxidase of the tulip tree was inhibited at pH 2.30 to pH 2.80, and increased in activity up to pH 6. He also found that the oxidase of magnolia was inhibited at pH 2.45 to pH 3.05 and increased with increasing pH value up to pH 5.70.

In the experiments to be described *Penicillium chrysogenum* was used. This plant was chosen because under the conditions of these experiments it does not produce sufficient alkali or acid (other than carbonic) to interfere with the results. Another reason which made it very good material to work with was its hardness (it was originally found growing on strong formalin solutions). *Aspergillus niger*, which has been used by the writer in previous work on respiration,¹³ produces a small amount of a non-volatile acid, for which reason it was not well suited to the method employed in the present investigation.

The way in which the fungus was grown, as well as the procedure employed in measuring the production of CO₂, and the method employed in calculating the rate are described in a previous paper by the writer.¹³

The pH of the solution in which the fungus was placed to determine the effect of hydrogen ion concentration was determined by means of indicators, using buffer solutions for comparison. For determining the concentrations around pH 1 to 4 tropeolin OO and methyl red were used, with buffers containing HCl and sodium citrate.¹⁴ For the concentrations at pH 7 to 9 phenolsulfonephthalein and phenolphthalein were employed and borate buffers were used for comparison.

The normal respiration was measured in 0.5 per cent dextrose solution in distilled water. To a similar solution sufficient acid or alkali was added to get the desired hydrogen ion concentration.

¹² Bunzell, H. H., *J. Biol. Chem.*, 1916-17, xxviii, 315.

¹³ Gustafson, F. G., *J. Gen. Physiol.*, 1919-20, ii, 17.

¹⁴ Sörenson, S. P. L., *Biochem. Z.*, 1909, xxi, 131.

NaOH was employed for the alkaline solutions. For the acid solutions H_3PO_4 and H_2SO_4 were used; to get rid of any volatile acid which might be present the acid was boiled for some time before using.

For preliminary work both H_3PO_4 and H_2SO_4 were employed, but as both of these acids gave practically the same results, when the H ions were present in the same concentration, the use of H_3PO_4 was discontinued. All the results here described were obtained by the use of H_2SO_4 .

In the experiments where the pH was less than 7 the apparatus described by Osterhout¹⁵ was employed. For the alkaline solutions this method could not be used, as the CO_2 neutralized the NaOH, and therefore the method described by Haas¹⁶ was employed. The method adopted here was to determine the time required to produce a given amount of CO_2 in normal respiration at pH 7.30 and then change the pH and determine the time required to produce the same amount of CO_2 .

This necessitated a determination of the change in pH produced by the same amount of CO_2 when added to neutral or alkaline solutions. This was accomplished in the following manner. Into a glass tube 10 cc. of a solution having a pH of 7.30 were put, and into a second tube 10 cc. of an alkaline solution; both solutions contained indicators. As soon as the tubes were filled they were stoppered and compared with standard buffers to get the exact pH. Into each tube one drop of the same solution of CO_2 in water was simultaneously introduced. The tubes were shaken and comparisons with standard buffers were again made in order to measure the change in pH. This procedure was repeated several times and the average of the changes in each solution taken as the change to be produced by the fungus. If for example the average of these changes was found to be from pH 7.30 to 7.10 in one solution, and from pH 8.92 to 8.68 in the other, standard buffers were made up for these values. Then the normal rate of respiration was determined by finding how long it took the fungus to change the pH from 7.30 to 7.10. Unless the rate remained constant for at least 20 minutes the material was rejected. (It had been found by experiment that after the respira-

¹⁵ Osterhout, W. J. V., *J. Gen. Physiol.*, 1918-19, i, 17.

¹⁶ Haas, A. R., *Science*, 1916, xliv, 105.

tion remained constant for 20 minutes it would keep this rate for hours provided food was supplied.) After a constant rate had been established, the material was transferred to the alkaline solution and the rate again determined by noting the time required to produce an equal amount of CO_2 ; *i.e.*, to change from pH 8.92 to 8.68.

The pH of the nutrient solution both before the spores were sown and after the mycelium had grown large enough to be used was found to be about 4. In no case was there any noticeable change during the growth. A few cultures were grown in a nutrient solution having a pH of 6 to 7. These cultures showed no difference in their behavior from those grown in a more acid medium. The medium on which the fungus was originally growing had a pH of 6 to 7. In all the experiments there was some change in the pH value of the solution containing the fungus. The average pH during the experiment is therefore taken in all cases as the pH of the experiment.

In the following account the rate of respiration at neutrality is arbitrarily designated as normal.

The preliminary work was started with solutions having a pH of about 4. With this hydrogen ion concentration there was no apparent effect. Next a solution of pH 2 was tried. Contrary to what was expected this concentration caused a considerable rise in the first half-hour followed by a fall below normal during the second half-hour.

After the preliminary experiments had given this result more careful experiments were made. The first concentration to be used was pH 1.35. This gave a rise of 20 per cent during the first 20 minutes, which was followed by a fairly rapid fall below normal. The second concentration to be used was pH 1.95. The rise in this case was more gradual than in the preceeding one and the fall was also much more gradual and did not fall nearly so much. A few experiments were also made with pH 1.10. These were similar to those with pH 1.35 except that the decrease was sharper and greater. Solutions of pH 1.70 gave results very much like those with pH 1.95.

A large number of experiments were made with pH 2.65. These results were rather variable. With only a few exceptions the rate of respiration was always at or above normal during the entire experiment. The experiments that gave the most divergent results were rejected, and from the remainder an average curve was constructed, which for 80 minutes did not fall below normal (Figs. 1 and 2).

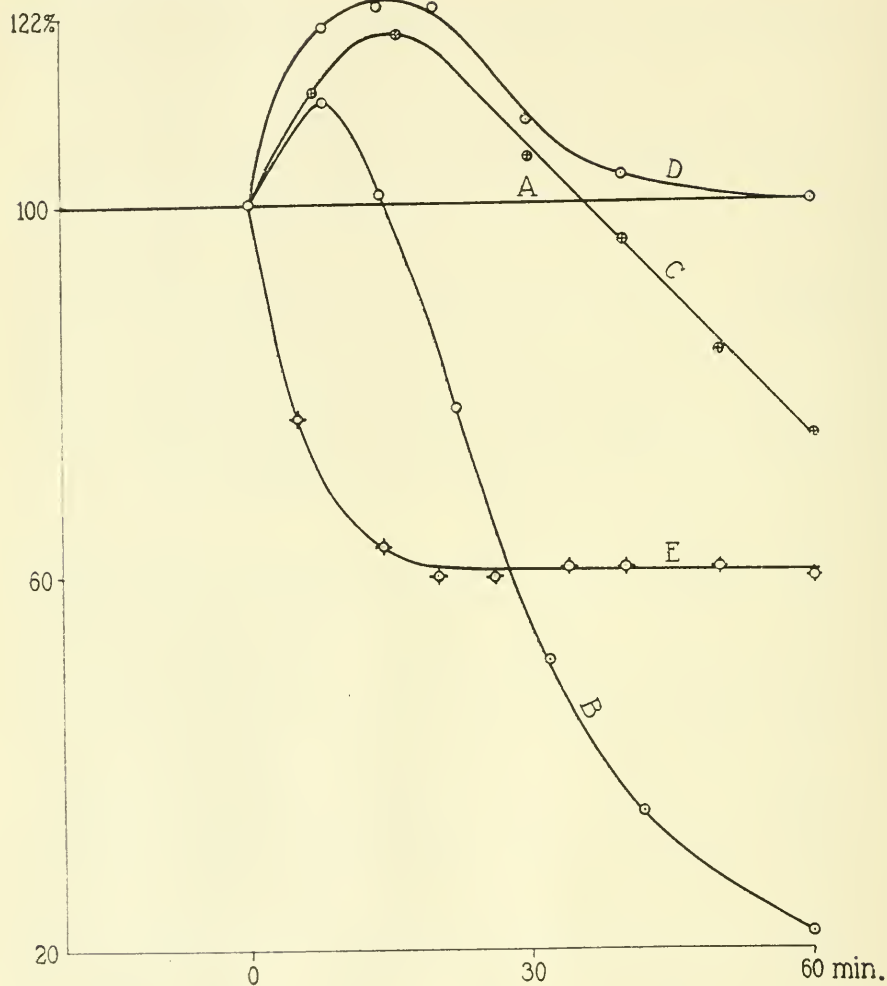
Rate of CO_2 production

FIG. 1. Respiration of *Penicillium chrysogenum* expressed as per cent of the normal (at pH 7). The material was put in the test solution at the point marked 0 on the abscissa and the line at the left of this point represents the normal respiration. Curve A represents the respiration in a solution having a pH of 7 (normal); Curve B in pH 1.10; Curve C in pH 1.95; Curve D in pH 2.65; and Curve E in pH 8.80. The normal rate represents a change in the indicator tube from pH 7.94 to 7.52 in from 2 to 3 minutes depending upon the amount of material used. The probable error was less than 5 per cent of the mean, except at the last point with pH 1.95 where it was 6.5 per cent of the mean.

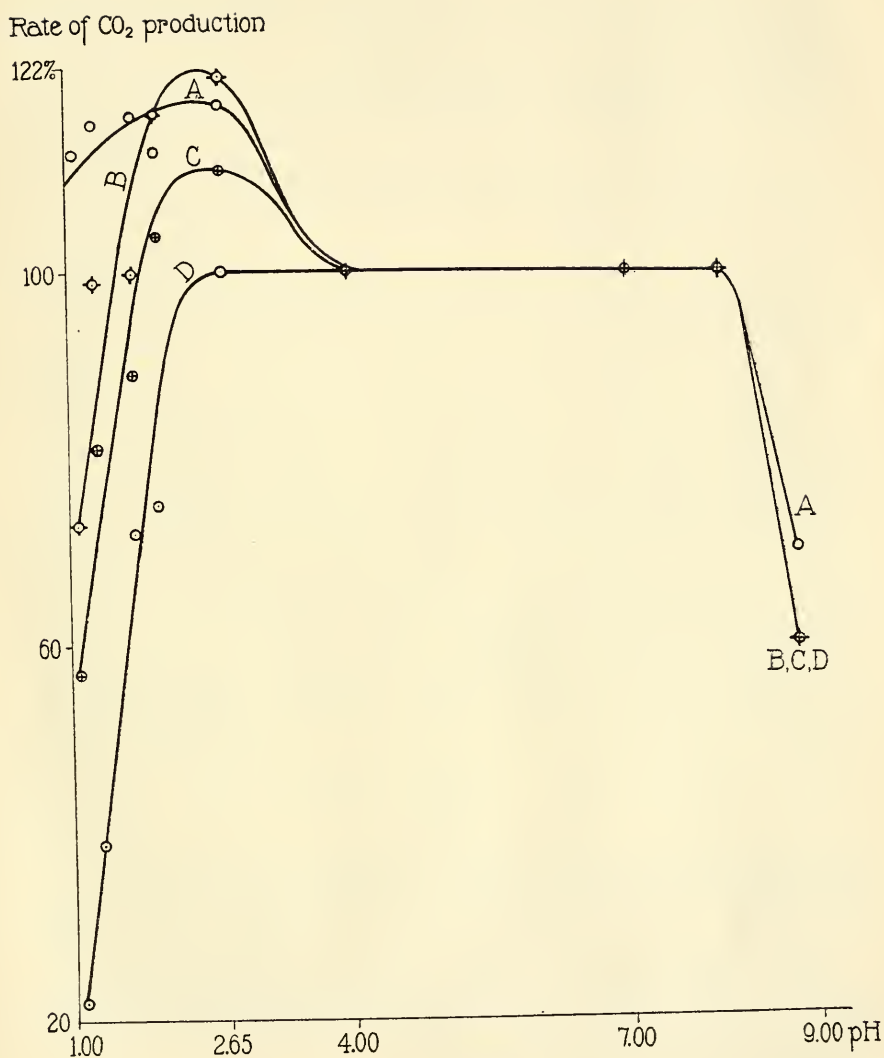


FIG. 2. Respiration of *Penicillium chrysogenum*. The curves show rate plotted against pH, and represent the same data as in Fig. 1. Curve A represents the respiration at the end of 8 minutes in the solution; Curve B at the end of 20 minutes; Curve C at the end of 30 minutes; and Curve D at the end of 60 minutes.

These experiments showed that in acid solutions the rate of respiration was increased at certain concentrations. The next step was to determine the effect of alkaline solutions.

The first concentration to be tried was pH 8. As it was found that this concentration had no effect on respiration the normal rate in the succeeding experiments was measured between pH 7.52 and 7.25 as the color change in phenolsulfonephthalein is much easier to determine at this range.

The next concentration chosen was pH 9. As phenolsulfonephthalein is not sensitive in this range phenolphthalein was used instead; this indicator is not toxic to the fungus. The concentrations were first standardized and it was found that the same amount of CO₂ caused a change from pH 7.52 to 7.25 and from 8.92 to 8.68. The time required for normal respiration to change the solution from pH 7.52 to 7.25 was determined; then the fungus was put in a solution slightly higher than pH 8.92 and the time required to change from pH 8.92 to 8.68 noted. It was found to be much longer than the normal, thus showing that respiration was decreased. Not only the first reading but all the succeeding ones showed a decrease, and at no time was there an increase.

When the pH value was less than 7 and there was a decrease to considerably below normal, no recovery back to normal (or nearly normal) followed, after the material had been replaced in ordinary nutrient solution at pH 4 or in sugar solution at pH 7. After the respiration had been depressed to 60 per cent of normal by pH 8.80 and kept there for an hour, there was nearly complete recovery when put back in sugar solution at pH 7. It is therefore evident that any considerable decrease produced by acid solutions is irreversible, while a similar decrease produced by alkaline solutions is reversible.

Some experiments have also been made by measuring the consumption of oxygen by the fungus. Winkler's method, as modified by Osterhout and Haas,¹⁷ was employed.

It was found that in a solution of pH 9 the fungus uses less than one-half as much oxygen as in a neutral solution, while in a solution of pH 2 the consumption of oxygen is nearly four times as great as

¹⁷ Osterhout, W. J. V., and Haas, A. R. C., *J. Biol. Chem.*, 1917, xxxii, 141.

in a neutral solution. These experiments confirm the data obtained by measuring the production of CO_2 , and show that in an acid medium the increase in oxygen consumption is greater than the increase in the production of CO_2 , while in alkaline solution the two are about the same.

In all cases control experiments were made with solutions containing no fungus but having the same pH values as the solutions containing the fungus.

The results indicate that the increased production of CO_2 in acid solution is due to respiration and not to the action of the reagent in setting free CO_2 previously stored in the tissue (in the form of carbonates).

The work of certain investigators might lead to the expectation that, in general, moderate concentrations of acid would decrease respiration, while moderate concentrations of alkali would increase it. It is evident that the organism here studied behaves in the opposite manner. This raises some interesting questions regarding the reactions in the organism which result in the production of CO_2 ; further investigation will be necessary to clear up these questions. It is possible that the results obtained from the study of *Penicillium* are connected with the fact that it grows best on an acid medium. In the experiments of the writer spores grew best on media with a pH value of from 4 to 6.

SUMMARY.

1. Variations in pH value between 4 and 8 produce practically no effect on the normal rate of respiration (the rate at neutrality is called normal).

2. Increasing the pH value to 8.80 causes respiration to fall to 60 per cent of the normal, after which it remains stationary for the duration of the experiment.

3. Decreasing the pH value to 2.65 causes a gradual rise and a gradual return to normal; at pH 1.10 to 1.95 the preliminary rise amounts to 20 per cent and is followed by a fall to below the normal.

4. The decrease in respiration brought about by solutions of a pH value of 1.95 or less are irreversible, while a similar decrease

which occurs at pH 8.80 is reversible, the rate coming back to practically normal after the material is replaced in a neutral solution.

5. Determinations by means of Winkler's method showed an increase in the consumption of oxygen in acid solutions and a decrease in alkaline solutions.

THE ANALYSIS OF NEUROMUSCULAR MECHANISMS IN CHITON.

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(Received for publication, May 3, 1920.)

From the standpoint of its grosser structure the nervous system of Placophorans is but poorly centralized, as evidenced by the absence of clearly defined ganglionic concentrations upon its principal strands. Histologically, the character of this nervous system is incompletely known. From the functional side, evidence has been secured pointing to the relatively diffuse type of nervous organization prevailing in Chiton as compared with some other mollusks (Arey and Crozier, 1919). It was desirable to make further inquiry as to the nature of the nervous organization of Chitons, especially as revealed through the actions of alkaloids. The species employed was *Chaetopleura apiculaia* Carp, (found at Woods Hole, Massachusetts).

The neuromuscular responses of Chitons comprise movements of various parts of the body, resulting from localized stimulations, and movements of the animal as a whole. In the latter category are included locomotion, suctional attachment to a substratum, and curling up movements.

The locomotion of *Chaetopleura*, like that of other Chitons (*cf.* Crozier, 1918-19), is accomplished by neuromuscular waves which travel from anterior end to posterior end over the surface of the foot. In this species, as in other cases, one pedal wave at a time usually appears on the foot, although in the larger specimens (20 mm. in length and over), two or three successive waves may be seen on the foot simultaneously.

Chaetopleura is negatively phototropic when exposed to light of 150 or more meter candles intensity; to light of less than 150 meter candles the older individuals are indifferent. An animal creeping in bright light ceases movement when the light is removed, but the shell surface is not sensitive to a decrease of light intensity.

The strong suctional attachment of Chitons, following stimulation, is effected by the girdle; the flexible girdle is pressed into contact with the substratum, and a subsequent slight arching of the body serves to produce a true "sucker" action quite independent of the activity of the foot. The surface of the foot does, however, suck locally (Parker, 1914), an area several millimeters square being sufficient to afford a suctional field adequate for the support of the whole body. An undisturbed *Chaetopleura* is supported in this way, for the girdle is then lifted permitting the entrance of respiratory water currents.

A *Chaetopleura* detached from its substrate and placed on its back undergoes, in the light, a pill-bug-like curling up. In weak light or in darkness, the animal after a short time opens out, twists longitudinally, and may succeed in righting itself. The ventral surface is quite sensitive to light, and to shading. *Chaetopleura* attached to a glass slip may be caused to release the attachment of the foot by the illumination of the ventral surface. Either increase or decrease of light intensity causes a *Chaetopleura* lying on its back to curl up more completely. By means of such changes of light intensity, produced on the ventral surface through the animal's own movements while beginning to "open up" after being curled, a more or less rhythmic succession of curling contractions may be maintained. The posterior end of a curled up *Chaetopleura* is commonly more sharply bent than the anterior, which thus overlaps it (as noticed also by Sampson, 1895). The girdle musculature is not strongly contracted when the Chiton is curled up.

The degree of longitudinal curvature of the body of a Chiton, and the degree of elevation of the girdle are each controlled by the relative tensions of antagonistic muscle groups (for structure, *cf.* Sampson, 1895; Plate, 1901). The important muscles are: (1) a median dorsal group, composed mainly of fibers running from the anterior edge of each shell plate to the ventral surface of the anterior region of the next anterior plate; (2) longitudinal muscles at either side of the body (flexor antagonists of the extensor muscles (1)); (3) the dorsal (extensor) and ventral (flexor) muscles of the girdle, set perpendicular to its margin. The contact irritability of the foot and the activities of these muscle groups were studied especially in relation to the effects of strychnine, nicotine, and curare.

Strychnine.

Chitons attached to the bottom of a glass dish were covered with sea water, and later strychnine sulfate in sea water was added to the desired concentration; or else the animals were immediately covered with strychnine solution. The effects secured depended upon the strychnine concentration and upon the time of action.

In strychnine with a concentration of 1:10,000, after about 3 minutes (at 19°C.) the girdle of a Chiton begins to turn upward; the foot ceases to attach to the substratum, and the whole body curls upward at both ends; usually the animal falls over on one side, and finally comes to lie more or less on its back because of lack of stable support on the curved margin of the girdle; it later curls in the more usual fashion, with the dorsal surface convex. Typical waves, frequently several at a time, appear in rapid succession on the foot, while the body is bent; and the proboscis is greatly extended. At no time does the animal respond to a sudden shading of its dorsal surface, although for a brief interval reactivity to a shading of the ventral surface is greatly enhanced; shortly thereafter this kind of irritability is obliterated. From this moment, that is after about 5 minutes exposure to strychnine, the Chiton responds to a sudden *increase* of illumination upon its ventral surface by a very decided further uncurling—whereas, in the non-strychninized animal, such stimulation induces prompt curling. This reversal of the usual reaction to increased illumination persists for about 15 minutes if the Chiton is at this point returned to sea water; it is lost if the animal continues in the strychnine solution for 24 hours, but even in this case it reappears during subsequent destrychninization in sea water.

The initial phase of dorsalward (opisthotonic) curvature, due to the contraction of the median longitudinal and oblique dorsal muscle bands, is succeeded by the strong contraction of the ventral girdle muscles and of the lateral longitudinal muscles, so that the body becomes curled as in the usual curling up maneuver. The dorsal shell plates are caused to separate slightly in this process, so that the intertegmental mantle between each two plates is exposed and open to stimulation by touching it with a pointed instrument. In the unstrychninized Chiton local stimulation of the mantle between two

plates results in the approximation of these two plates, thus covering the spot irritated; this reaction is in no way modified under strychnine (even at 1:4,000 concentration). The girdle, also, responds in an entirely normal fashion to local tactile activations, as do the ctenidia. Another form of response which remains unaltered under strychnine is the dorsalward contraction of the ctenidia against the roof of the ctenidial channel when a spot on the same side of the foot is touched. The surface of the foot itself exhibits an interesting modification of its responses; the foot ordinarily responds to the contact of a surface, sufficiently large with respect to the foot, by a positive suctional attachment (the adhesion of the foot being due to the activity of many minute suctional fields over its surface); but under strychnine, the foot puckers away from the contact of a surface of any size—its positive stereotropism suffers reversal. In the non-strychninized Chiton the touch of a fine-pointed rod causes merely a localized but rather deep puckering of the foot. Under strychnine, touching one small point leads to a widespread retraction.

With more concentrated solutions (1:4,000) these effects develop more promptly. The body becomes at first greatly arched, the back convex, with some convulsive trembling, but the girdle does not curl ventrally. If touched on the back, the animal usually ceases to adhere to the bottom and curls up, armadillo fashion, remaining so for some minutes. Ultimately, even in strychnine of low concentration (1:12,000), the Chitons pass into a paralyzed state, with all muscles contracted, the girdle curled ventralward, the two ends of the body more or less approximated ventrally.

The significant features of these results are: (1) The reversal by strychnine of the suctional attachment of the foot;¹ (2) the neuromuscular reversals exhibited in the dorsalward curvature of the girdle and of the body as a whole, probably due to the effects of impulses originating in those parts at the time in contact with the substratum; (3) the reversal of the response to increased illumination of the ventral surface; (4) the fact that certain responses seem to remain

¹ This is paralleled by Moore's (1916-17) finding with the tube feet of strychninized starfish.

relatively uninfluenced by strychnine (movements of the ctenidia, and of the dorsal plates when the intertegmental mantle is touched).²

In the strychninized Chiton sensory impulses originating on the ventral aspect of proboscis, foot, and girdle may be regarded as causing contraction of *extensor* (dorsal) muscles of the girdle and of the body (*median dorsal longitudinal* and *oblique dorsal muscles*). This effect may be accentuated by touching the back of the Chiton. Such impulses normally lead to the contraction of *flexor* muscles (*ventral* muscles of the girdle; *lateral longitudinal muscles* connecting the shell plates) which results in a closer contact between the Chiton and its substratum.

Reversal of the response to increased illumination of the ventral surface, involving the muscle groups mentioned, operates in the same manner. This seems to be the first recorded instance of experimental reversal of the sense of a response dependent upon "differential sensitivity."

There is a detectable degree of independence in the movements of strychninized girdle and body. Thus at certain stages a touch on the back may cause the girdle to bend ventrally, while the extensor muscles of the back contract; in other instances the back may be arched, while the girdle bends dorsally. The type of local action here indicated is further illustrated by the behavior of Chitons with the pallial nerve strands cut and with the proboscis region isolated from the foot by a transverse cut. Such preparations do not differ noticeably from intact animals in their responses to strychninization.

² In a number of instances Chitons returned to sea water after having been in strychnine solution for a number of hours were found to pour out great volumes of sperm, at a certain stage in the progress of recovery. The observation is of interest in connection with the view (Crozier, 1920) that the shedding of sperm by male Chitons, although in normal fertilization preceding the liberation of eggs, is in reality induced as a sensory response to substances emanating from females; in these experiments the proper internal concentration of strychnine for the release of the nervous machinery controlling sperm discharge seems to have been established during the outward diffusion of the drug from the Chiton into sea water.

Nicotine.

Attached *Chaetopleura* immersed in 1:25,000 solutions of nicotine (Kahlbaum) within 3 minutes release their suctional attachment to a surface, and curl ventralward rather sharply at both extremities. The proboscis fold is commonly much protruded, covering the anterior end of the foot. The girdle is curled inward. In this tightly curled condition the Chiton quickly passes into paralysis. After 15 minutes in nicotine solution, recovery of normal movements is effected within an hour following return to sea water. At 1:1,000 concentration the nicotine effect is almost instantaneous. After the first few seconds in nicotine solution the ordinary responses to changes in illumination are obliterated.

Nicotine primarily induces the contraction of *flexor* muscle groups (ventral girdle muscles, lateral shell muscles), whereas in the early stages of strychninization the extensor antagonists of these muscles are the most readily contracted.

A further peculiarity of the nicotine action consists in its selective nature. The anterior end of the Chiton is almost invariably more strongly flexed than the posterior. When curled up in the absence of nicotine, the posterior end is the more flexed, the anterior end folding over it. When the body has been partially transected in such fashion that the soft tissue of the foot is separated from the proboscis fold, while the dorsal muscle bands are left in continuity, it is found that nicotine induces as promptly as before the general flexion of the girdle, while the foot and shell muscles remain for a long time unaffected. The action of strychnine on such a preparation, already noted, is quite different. Thus the "cerebral" region of the nervous system of Chiton, despite the absence of pronounced cephalization, exhibits nevertheless a certain degree of selective reactivity with nicotine, homologous with that demonstrated by Moore (1918-19) for the cerebral ganglia of the squid.

Curare.

Immersion of a Chiton for 3 hours in a saturated emulsion of active curare has practically no detectable results on the animal's movements. Detached and partially curled up *Chaetopleura* dropped

into curare solution tend on the whole to remain curled up for a longer period (even in the dark) than is the rule in sea water, but, even after 5 hours in curare, recovery in sea water is quite prompt. The isolated foot of a Chiton, if exposed to curare, does not go into spasms of contraction. Curare has a quite different effect on the foot and on the whole animal in the case of gastropods, usually inducing convulsive contractions. Even after 7 hours in curare the tendency to curl up is not materially enhanced; the foot is usually crinkled somewhat, but not decidedly, and the girdle is not strongly contracted. It is of interest to note that curare has practically no action on neuromuscular coördination in flatworms.³

In comparison with the definite curvatures of body and girdle characteristically induced by nicotine and in early stages of strychninization, it is found that in quinine solutions the curvature of the mantle is quite irregular, while the body may be curled to varying degrees.

SUMMARY.

1. The degree of curvature of the body and of the girdle of a Chiton is determined by the activity of antagonistic muscle groups. At a certain early stage in the strychninization of a Chiton the reciprocal inhibition involved in the natural use of these muscle groups is reversed, such that extensor muscles, rather than, as normally, flexor muscles, contract as the result of stimulation. This condition involves a reversal, under strychnine, of the normally positive stereotropism of the foot, and of the usual response of the mollusk to an increased illumination of its ventral surface. Strychnine reversal of this character is not a matter of the relative strength of the opposed muscle groups, for the flexor muscles are the more powerful and are the ones always shortened in tetanic contraction.

2. Nicotine, in contrast to strychnine, primarily induces contraction of flexor muscles. Its effects, moreover, are in a degree selec-

³ Vles (1907) thought the antero-posterior direction of the pedal waves in *Acanthochites* suggestive of a worm-like neuromuscular organization, more primitive than that in the foot of gastropods, where the waves are often *direct*; but the retrograde waves producing locomotion in polyclads (Crozier, 1918) seem to be rather more complex than in Chitons (Crozier, 1918-19).

tive, being notably exerted on "cerebral" nervous structures. Curare is devoid of characteristic action on the neuromuscular responses of Chiton.

3. The chemical organization of the neuromuscular organs of Chiton, as far as revealed by these tests, corresponds to a more simple condition than is inferred for gastropods. In particular, the behavior with respect to curare resembles more that of the neuromuscular apparatus of flatworms.

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THE KINETICS OF THE ACTION OF CATALASE EXTRACT FROM MARINE ALGÆ, WITH A NOTE ON OXIDASE.

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(Received for publication, March 1, 1920.)

There exists, as far as we know, no literature on the catalase of marine algæ. The work done on oxidase and peroxidase is scanty and incomplete (Reed, Atkins, and Duggar and Davis). We have started a preliminary investigation of the catalase, peroxidase, and oxidase of marine algæ. The present paper deals only with the enzymes of *Ulva tæniata* Setchell and Gardner, although we worked with different forms. *Ulva* contains the strongest oxidase, according to Reed. This fact, and the abundant occurrence of *Ulva* at Pacific Grove, led us to study this form more particularly.

Our work is only preliminary, for as yet we have not considered the daily variations and other features of the behavior of the enzyme. We hope to publish soon a more physiological paper on the same question. We worked with one "clan" of *Ulva*, placed in an aquarium with running sea water, the temperature of which was practically constant throughout our experiments. We always worked upon the youngest plants, but the "physiological error" will remain in our work a source of inevitable inaccuracies.

Catalase.

General Remarks.—The alga contained a rather active catalase, as qualitative experiments have shown. To determine its strength we used a manometrical method, elsewhere described by us. A suction flask was connected with a self-recording manometer by a ground joint. This joint permitted thorough shaking of the flask. The suction flask was closed by a ground stopper to which the peroxide container was sealed on the inside. On inverting the flask, the peroxide would pour into the fluid in the flask and the reaction would

begin. The curves made on millimeter paper by the automatic writer could be calculated immediately. The advantages of this method are: (1) accurate determination of the beginning and the end of the reaction and of the reaction *time*; (2) accurate determination of the strength of the peroxide; (3) accurate and quick measurement of reaction velocities in very short intervals; *e.g.*, every 5 seconds.

Fig. 1 is an autogram showing the effect of the action of 3 cc. of 3 per cent peroxide on 3 gm. of ground *Ulva* thallus in a slightly alkaline medium. The ordinates are pressures in millimeters. The abscissæ are 14 seconds per millimeter. We used Braun-Knecht-Heimann's c. p. 3 per cent peroxide which contains still acetanilide and acid. We neutralized the peroxide with 0.1 N sodium carbonate solution.

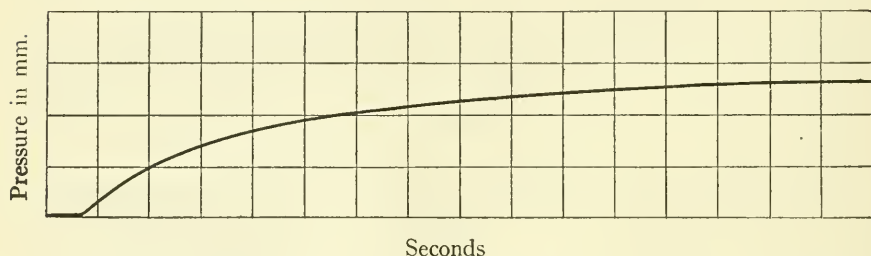


FIG. 1. Autographic record of the effect of 3 cc. of 3 per cent peroxide on 3 gm. of fresh, ground thallus of *Ulva*.

The activity of the catalase in *Ulva* is not very great. If we compare it with the activity of hemp leaves, we get

3 gm. of ground <i>Ulva</i>	4 cc. of H_2O_2 + 0.4 cc. of 0.1 N Na_2CO_3
Average.....	$K = 521 \times 10^{-5}$
3 gm. of ground hemp leaves...	4 cc. of H_2O_2 + 0.4 cc. of 0.1 N Na_2CO_3
Average.....	$K = 7,020 \times 10^{-5}$

The *hemp* is about fourteen times stronger than the *alga* in its catalytic activity.

Before giving a detailed description of the experiments, we wish to emphasize the fact that, however simple the method described may seem, we met with great difficulty in carrying it out. We discarded most of our experiments because we were not sure of their

reliability. Only smooth, finished curves were used for calculation. These were produced in experiments in which the ground plant was mixed thoroughly with the peroxide. In connection with these remarks a quotation of Pickering is given:

"The application of the graphic method requires a great amount of care and close attention to experimental and other conditions, and it is to be feared that hurried use of it by those who have not taken the trouble to master the necessary details, or to acquire the requisite amount of skill, may bring it into undeserved disrepute."

It is almost unnecessary to say that we used for our calculation the formula of van't Hoff

$$K = \frac{1}{0.434t} \log \frac{a}{a-x}$$

In the few cases to which this formula did not apply we used others. All the determinations were made at $\pm 17^\circ\text{C.}$ ($16-18^\circ\text{C.}$). The temperature of the sea water was invariably 15°C.

Catalase of the Undamaged Plant.—Catalase is an intracellular enzyme and is supposed to adhere to the cells with great tenacity. The extent to which the material is ground has a marked effect upon the result. Long and thorough grinding in a mortar reduces the alga to the consistency of an unguent. With the material in this condition we obtained our best results. At first we washed off the adhering sea water with distilled water before the grinding, thinking that the salt water might injure the enzyme. We were very much surprised, however, to find that these preliminary washings reduced the enzyme strength one-half, whereas a treatment with sea water had no effect at all.

3 gm. of *Ulva* + 3 cc. of unneutralized peroxide, ground without washing, gave the following.

$K \times 10^5$ after.				Average. $K \times 10^{-5}$
138 sec.	276 sec.	414 sec.	552 sec.	
275	270	256	254	264

K decreases because of the acidity of the peroxide.

3 gm. of *Ulva* + 3 cc. of unneutralized peroxide, ground after washing twice in distilled water, gave the following.

$K \times 10^5$ after.				Average. $K \times 10^{-5}$
138 sec.	276 sec.	414 sec.	552 sec.	
128	110	86	81	101

K decreases because of the acidity of the peroxide.

We could offer two explanations of this fact: (1) The distilled water destroys the enzyme. (2) The enzyme migrates into the wash water. To decide which hypothesis was true, we made a catalase determination with the wash water. We soaked 20 gm. of *Ulva* in 200 cc. of distilled water for 40 minutes. To 4 cc. of this wash water we added 3 cc. of peroxide solution. The wash water contained catalase.

$K \times 10^5$ after.				Average. $K \times 10^{-5}$
138 sec.	276 sec.	414 sec.	552 sec.	
40	33	31	26	33

On the assumption that there is proportionality between K and enzyme quantity for 3 gm. of plant, calculation gave a yield of catalase of 130×10^{-5} as average K to the distilled water. We can say, therefore, that the plant lost one-half its catalase to the surrounding water. We obtain

Washed plants: $K \times 10^5$	= 100
Wash water.....	= 130
	230

Unwashed plants had a $K \times 10^5$ of 264 which is a fairly close approximation. After 1 hour and 40 minutes washing, the wash water contained no more catalase than after 40 minutes. The curve obtained was identical with the curve analyzed above. Wash water, in which the plant remained for only 3 minutes, showed no catalytic activity.

We then put 20 gm. of plants from the aquarium in 25 cc. of distilled water and let it stand over night. The next morning 4 cc. of the fluid gave

$K \times 10^5$ after.				Average K .
138 sec.	276 sec.	414 sec.	552 sec.	
139	109	96	85	107

which gives a yield of catalase of $K \times 10^5 = 85$ from 3 gm. of plants. The source of the above tables is the autographic record shown in Fig. 2.

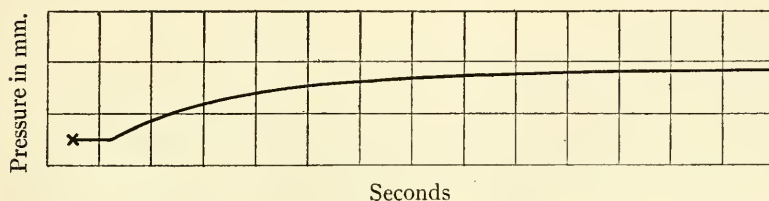


FIG. 2. Autographic record of presence of catalase in distilled water used to wash *Ulva*.

The enzyme is more or less destroyed by long standing, and we can conclude with certainty that approximately *one-half the total amount of catalase passes out into the wash water within the first 40 minutes of washing.*

There is, as far as we know, only one paper dealing with a similar question. Lyon has noted similar behavior with echinoderm eggs. He found that in these eggs the catalase activity was doubled after fertilization. The increase in activity started about 3 minutes after fertilization. Lyon discusses several possible causes of this fact and finds that neither the sperm nor the "increase in activity" is responsible for the change but on the other hand a difference in the permeability of the cell membrane. This is consistent with the theory of fertilization set forth by Loeb, Warburg, and others. Lyon was able, by placing the echinoderm eggs in distilled water, to *double the catalase activity in unfertilized eggs*; in other words, they gave the same reaction as the fertilized eggs. Lyon seems not to be aware of

the fact that the catalase was suspended in the water. In connection with our own experiments we tried to determine the facts microscopically.

Experiment 1.—The damaged piece of thallus was placed in sea water + a trace of H_2O_2 . Bubbles of oxygen originated only at the place where the piece was cut. After a long time there was a very feeble activity on the surface ceasing after about 2 hours.

Experiment 2.—A similar piece of thallus was placed in distilled water + a trace of H_2O_2 . Oxygen originated immediately at the place where the piece was cut. After about 3 minutes there was a strong reaction on the whole surface, which lasted for about 50 minutes.

There are two conclusions possible, which might explain these facts: (1) catalase goes out by exosmosis; (2) catalase goes out through the burst cell wall.

While the second assumption may be possible, there is one fact that can be explained in favor of the first assumption. If the cell bursts, there is no reason why any catalase should remain in the cell, as the enzyme is soluble in water. A fact that reminds one of exosmosis is the short space of time within which the emigration of the catalase is completed. It is improbable, on the other hand, that large molecules diffuse out of the cells in which they occur. We may suspect, therefore, that this enzyme at least is a simpler compound than most of us have thought hitherto.

Influence of Substrate Concentrations.—We considered one series of experiments done with homogeneous material under the same conditions. 3 gm. of *Uva* were ground in a mortar to a very fine consistency with 15 cc. of distilled water. The peroxide was previously neutralized by 0.1 cc. of 0.1 N sodium carbonate solution per cc. We found

Peroxide.	End-pressure.	$K \times 10^5$ after.					Average. $K \times 10^5$	Reaction time.	
		138 sec.	276 sec.	414 sec.	552 sec.	690 sec.		Found.	Calculated.
cc.	mm.							sec.	sec.
4	44	306	298	295	299	306	299	745	744
3	32	342	333	353	366	361	337	573	558
2	21	438	408	461	514 (?)	437	423	372	372
1	10	667	689	—	—	—	678	207	186

Peroxide quantities are proportional to reaction time, as the last two columns show. No linear relation exists, however, between the former and the reaction velocity. Fig. 3 shows that this relation can be expressed by a continuous curve which is not exponential, as the following calculation indicates.

Peroxide.		K^1	$K^{1.5}$	$K^{1.8}$	K^2
cc.					
4	$\frac{K}{4}$	100	100	100	100
3	$\frac{K}{3}$	56.5	57.4	62.9	64
2	$\frac{K}{2}$	47.1	57.4	62.2	65
1	$\frac{K}{1}$	53.1	83.1	107	123

There seems to be no simple connection between peroxide quantity and reaction velocity. The laws derived by Schutz-Borissow, Issajew, and Herzog do not hold here.

This is otherwise with enzyme quantity. Three curves, run with 3 cc. of neutralized peroxide and different amounts of enzyme gave

Enzyme.	$K \times 10^5$	$K \times 10^5$ Calculated.*
2 gm. of plant.	256	254
3 " " "	380	380
4 " " "	506	508

* K calculated after 414 sec.

According to the low strength of the enzyme, the peroxide is very much in excess, and therefore the reaction velocity is proportional to the enzyme concentrations, not to the peroxide concentrations. Senter found exactly the same to be the case with blood catalase (see also the more recent paper by Yamasaki). The values obtained with hemp gave a proportionality to both peroxide and enzyme quantity. This is probably due to the great strength of the hemp catalase. However, it is remarkable that the reaction velocity still remains in a linear relation to the peroxide quantities. We have no

doubt that this relation will exist also with different enzyme quantities. Reaction time is a very valuable index for enzyme determination, and the fact that Duclaux and Went are the only authors who used this index in physiology shows us that it is very little known. The catalase strength is often determined by measuring a pressure after a certain time, but this method has no value for quantitative determinations.

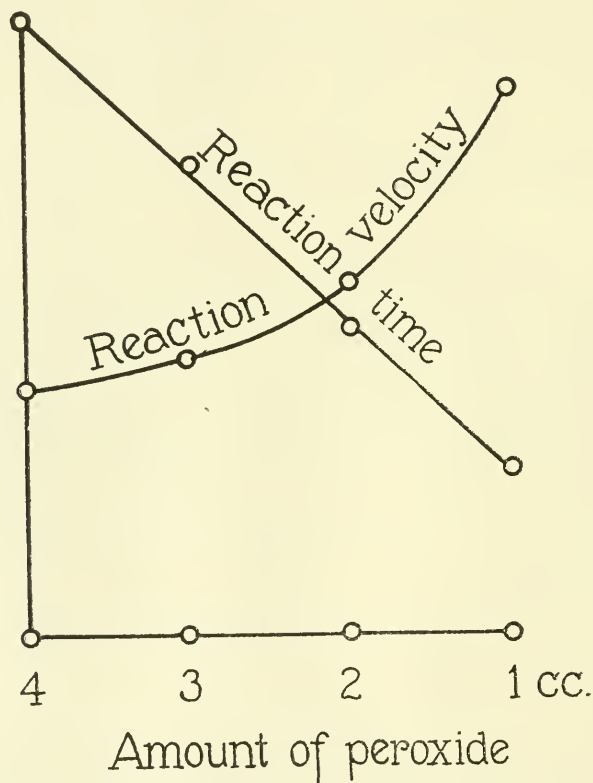


FIG. 3. Relations of peroxide quantity, reaction time, and reaction velocity.

Influence of External Conditions.—Under certain precautions the reaction follows the monomolecular line. For example, 3 gm. of *Ulva* were ground as fine as possible in a mortar with 15 cc. of distilled water. After reaction with 4 cc. of peroxide and 0.4 cc. of 0.1 N Na_2CO_3 solution we calculated the resulting curve.

Time.	$a - x$	$\log \frac{a}{a - x}$	$\frac{2.31}{t} \log \frac{a}{a - x}$
<i>sec.</i>			
138	29.0	1.183	306×10^{-5}
276	19.5	0.353	298×10^{-5}
414	13.0	0.527	295×10^{-5}
552	8.5	0.715	299×10^{-5}
690	5.5	0.904	306×10^{-5}
828	3.8	1.063	296×10^{-5}
966	2.4	1.272	304×10^{-5}
Average			$(300 \pm 9) \times 10^{-5}$

$a = 44$ mm.

The reading error, 0.2 mm., gave the following result in the first K :

138 29.2 0.178 297×10^{-5}

The deviations in K are therefore due to reading errors, and the reaction follows the monomolecular line.

We studied the influence of different amounts of sodium carbonate with different 3 gm. lots of *Ulva*. We found

	$K \times 10^5$ after.				Average $K \times 10^5$
	138 sec.	276 sec.	414 sec.	552 sec.	
3 cc. of H_2O_2 + 1.6 cc. of Na_2CO_3 (neutral to phenolphthalein)	338	356	360	349	351
3 cc. of H_2O_2 + 100 cc. of Na_2CO_3 (alkaline)	251	219	215	176	215
3 cc. of H_2O_2 + 1,000 cc. of Na_2CO_3 (alkaline)	165	155	151	149	155

Excess of alkali causes a marked decrease in reaction velocity in subsequent repetitions of the reaction. Perhaps the enzyme is destroyed by the carbonate. Therefore we tried to apply the formula of Schmidt-Nielsen (shaking in activation of rennet) $\frac{dx}{dt} = k(a - x)^{\frac{3}{2}}$ in the modification given by Herzog

$$K = \frac{\sqrt{a} - \sqrt{a - x}}{t \sqrt{a(a - x)}}$$

	\sqrt{a}	t	$\sqrt{a-x}$	$\sqrt{a(a-x)}$	$K \times 10^5$
3 cc. of H_2O_2 + 100 mg. of Na_2CO_3 .	5.25	276	3.87	20.4	245
		414	3.39	17.9	251
		552	3.00	15.8	254
		690	2.74	14.4	256
		828	2.55	13.4	244
		966	2.34	12.4	243
Average					$(249 \pm 7) \times 10^{-5}$
3 cc. of H_2O_2 + 1,000 mg. of Na_2CO_3 .	5.25	276	4.18	21.9	177
		414	3.8	19.9	176
		552	3.47	18.3	174
		690	3.16	16.6	182
		828	2.95	15.7	177
		966	2.72	14.3	184
Average					$(178 \pm 7) \times 10^{-5}$

So we see that the formula of Schmidt-Nielsen is valid for the case of excess carbonate.

Unneutralized peroxide was harmful, as the following experiment shows.

3 gm. of *Ulua* + 3 cc. of unneutralized H_2O_2 and 15 cc. of distilled water.

$K \times 10^5$ after.				Average. $K \times 10^5$
138 sec.	276 sec.	414 sec.	552 sec.	
275	270	257	256	265

The summary of this experiment is given in Fig. 4. The point 0, in the figure, "neutral to phenolphthalein," seems to be the optimum point for catalase. (For similar lines see Bredig and von Berneck and Jacobson's work.)

As described above, the water in which undamaged plants remained for a certain time contained catalase. This catalase did not follow the logarithmic curve.

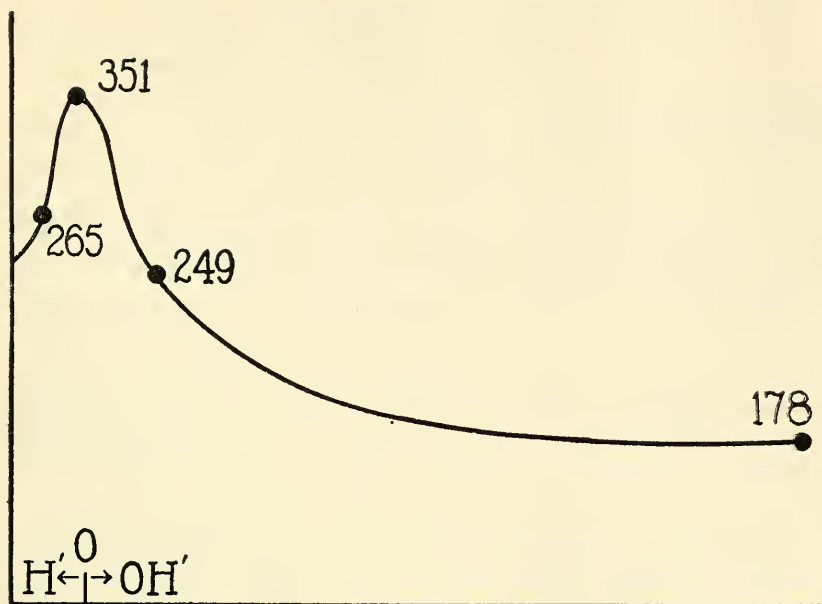


FIG. 4. Graphic indication of the reaction velocities with varying amounts of Na_2CO_3 .

	t	$K \times 10^5$	$K^2 \times 10^5$	$K^2 \times 10^5$	$K^3 \times 10^5$
20 gm. of plant in 25 cc. of water.	138	139	134	130	129
	276	109	109	117	123
	414	96	99	101	120
	552	85	91	95	126
Average					$(124 \pm 5) \times 10^{-5}$

Calculation following Schmidt-Nielsen's formula (K as in Column 4) gave no better result. Next we tried $\frac{dx}{dt} = K(a - x)^2$ (as in Column 5)

and $\frac{dx}{dt} = K(a - x)^3$ (as in Column 6). The latter gave a constant series. The destruction of the enzyme is probably due to other causes (substances washed out by exosmosis (?)).

	t	$K^1 \times 10^5$	$K^2 \times 10^5$	$K^3 + 10^5$
20 gm. of plant in 200 cc. of water.	138	40	38	43
	276	33	35	38
	414	31	33	35
	552	26	29	31

However, this assumption does not hold, as the above table shows. Here the dilution is eight times greater than in the former case. The formula $\frac{dx}{dt} = K(a - x)^3$ does not apply here. It is obvious that the *dilution* is the factor which combines its injurious effects with the chemical influences. Herzog remarks that inactivation of enzymes is the quickest in dilute solutions according to the principle of Willard Gibbs. It is known in breweries that the diastase is resistant in concentrated malts, but is quickly destroyed in dilute malt.

Conclusions as to Catalase.—*Ulva taniata* Setchell and Gardner contains a catalase which adheres to the cells to a certain extent, but may, mechanically or by exosmosis, pass into the surrounding medium. This catalase is able to act in a neutral medium on peroxide, the reaction being monomolecular. In an alkaline medium the catalase decomposes peroxide following the formula of Schmidt-Nielsen

$$K = \frac{\sqrt{a} - \sqrt{a - x}}{t \sqrt{a(a - x)}}$$

The reaction time varies in a linear relation with the concentration of the peroxide. Reaction velocity and enzyme concentration vary in a linear relation. Reaction velocity and peroxide concentration vary in a continuous non-linear and non-exponential relation. Since the peroxide is in excess of the feeble catalase, these facts are in agreement with the data of Senter on blood catalase. On dilution the enzyme is destroyed, following highly exponential lines

$$\frac{dx}{dt} = K(a - x)^3$$

or even

$$\frac{dx}{dt} = K(a - x)^4$$

Oxidase and Peroxidase.

Oxidase.—Pyrocatechine is quickly oxidized in the presence of ground *Ulva*. 10 gm. of grindings, 200 mg. of pyrocatechine, and 25 cc. of water gave in 20 hours in the manometer a negative pressure of 2 mm. 5 gm. of grindings, 300 mg. of pyrocatechine, and 25 cc. of water gave in 20 hours in the manometer a negative pressure of 3 mm. These are indications that *Ulva* contains an oxidase. Reed found in *Ulva* an oxidase which acted on guaiacol, pyrogallol, hydrochinon, paraphenyldiamine, and α -naphthol.

To determine whether the reaction of the pyrocatechine was due to the action of the plant we prepared two test-tubes; A, 100 mg. of pyrocatechine and 10 cc. of distilled water, and, B, 100 mg. of pyrocatechine, 5 cc. of distilled water, and 5 cc. of *Ulva* extract. B gave a strong red color after 1 hour; A remained light yellow.

We were not able to get an appreciable reaction with guaiacol, paraphenyldiamine, or α -naphthol, or a combination of the last two substances.

100 mg. of pyrocatechine were oxidized in 17 hours by 1 cc. of extract to the same color as 0.2 cc. of 0.1 N potassium permanganate would give it.

100 mg. of pyrogallol were oxidized in 17 hours by 1 cc. of extract to the same color as 1.75 cc. of potassium permanganate would give it (dark brown). A control experiment without enzyme gave a light color (0.25 cc. of potassium permanganate).

100 mg. of guaiacol or 100 mg. of phenol were not oxidized in 17 hours.

In conclusion we may say that *Ulva* contains a rather strong oxidase, active on pyrocatechine and pyrogallol, inactive on guaiacol, phenol, paraphenyldiamine, and + α -naphthol.

Peroxidase.—We tried two peroxidase tests. One, the luminescence of pyrogallol + H_2O_2 (Harvey) failed under the different circumstances. The other, the action of plant juice on ring compounds + H_2O_2 succeeded only in a faintly alkaline medium. *We were able to obtain the same reactions without enzyme in alkaline solution.* Therefore, we do not feel justified in concluding that a peroxidase is present. For example

200 mg. of hydrochinon + 3 cc. of H_2O_2 give a feeble red color.
200 " " " + 0.4 cc. of 0.1 N Na_2CO_3 give a yellow color.
200 " " " + 3 cc. of H_2O_2 + 0.4 cc. of Na_2CO_3 give a strong orange color.

Addition of plant juice has no apparent effect. Similar results were obtained with paraphenyldiamine, pyrocatechine, and resin of guaiacol.

In connection with this fact we may mention the work of Welter on the peroxidase of tea. Welter found that sand (sea sand of Merck, purified) + H_2O_2 gave a peroxidase reaction with ring compounds.

SUMMARY.

Ulva taniata contains oxidase. The existence of peroxidase is not proved.

We wish to express our gratitude to Dr. Walter Fisher, Director of Hopkins Marine Station of Stanford University, for his hospitality and valuable help, and to Dr. N. Gardner, of the University of California, for determination of the material with which we worked.

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QUANTITATIVE LAWS IN REGENERATION. II.

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(Received for publication, May 21, 1920.)

I.

In a preceding paper¹ it has been shown that, when a piece of stem inhibits the production of roots and shoots in a leaf of *Bryophyllum calycinum* connected with it, the stem gains in mass and this mass equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem. On the basis of this fact it was suggested that the inhibitory influence of the stem upon the formation of roots and shoots in the leaf is due to the fact that the material available for this process naturally flows into the stem.

In these experiments the quantity of roots formed had not been measured directly but had been calculated on the assumption that the dry weight of the roots formed is on the average 42 per cent of the dry weight of the shoots formed in the same leaves. Since this experiment seems to be crucial for the answer to the question why the leaf does not form shoots or roots as long as it forms a part of a normal plant it seemed advisable to make a direct determination of the mass of roots formed by an isolated leaf. Five new sets of experiments, as a rule with eight pairs of sister leaves, were made. The method was the same as that described in the first paper.¹ Table I gives the dry weight of the organs. The experiments lasted about 1 month; if they last too short a time the error in measuring vitiates the result and if we wait too long another complication arises inasmuch as the leaves of the shoots formed become too large and contribute too much material for further growth and regeneration.

¹ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 297.

In Table I, a is the dry weight of roots and shoots formed by the isolated leaves without stems; b is the dry weight of the half stems cut off from these leaves at the beginning of the experiment; a_1 is the dry weight of roots and of shoots formed by the sister leaves left in connection with a piece of half stem. b_1 is the dry weight of the latter half stems at the end of the experiment. According to our theory the inhibitory effect of this piece of stem left in connection with a leaf on the production of roots and shoots by the leaf should be due to the fact that naturally the material (or the greater part of

TABLE I.
Dry Weight of Roots, Shoots, and Half Stems.

Experiment No.	Duration.	a		b	a_1		b_1	$\frac{a}{a_1 + b_1 - b}$
		Roots.	Shoots.		Roots.	Shoots.		
	<i>days</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
1	30	0.169	0.412	0.474	0.039	0.067	1.054	$\frac{581}{686} = 0.85$
2	33	0.133	0.408	0.426	0.062	0.158	0.753	$\frac{541}{547} = 0.99$
3	33	0.127	0.509	0.415	0.045	0.114	1.034	$\frac{636}{778} = 0.82$
4	30	0.127	0.438	0.563	0.053	0.143	1.029	$\frac{565}{662} = 0.85$
5	30	0.101	0.416	0.422	0.043	0.162	0.823	$\frac{517}{606} = 0.85$

the material) used for root and shoot production in the leaf flows into the stem and is utilized here for growth. We should therefore expect to find that the dry weight of the half stem (b_1) left in connection with the leaf should increase as much as or even more than the difference in the dry weight of the roots and shoots produced in the two sets of sister leaves amounts to. If a is the mass of shoots and roots produced by the leaf isolated completely from the stem, a_1 the mass of roots and shoots produced by the sister leaves left in connection with the half stem, b the mass of the half stems at the beginning of the

experiment, and b_1 the mass at the end, we should expect to find that $b_1 - b \geq a - a_1$ or that $\frac{a}{a_1 + b_1 - b} \geq 1$. Table I shows that this is correct. The average ratio of $\frac{a}{a_1 + b_1 - b}$ is 0.87.

When a leaf is connected with a stem which prevents regeneration in a leaf the inhibitory effect is therefore due to the fact that the material available for the regenerative growth in the notches of the leaf flows into the stem. In fact 14 per cent more dry weight goes from the leaf into the stem than would go into the growth of the regenerating leaf. For some reason the flow of sap into the regenerating parts in the notches of a leaf seems to be less complete than the flow of available material into the stem.

II.

The material which goes into the stem is used for different purposes; it may cause the growth of an axillary shoot in the stem as in Fig. 1. Part of it causes an increase in the mass of the stem. The stippled lines in Fig. 1 indicate part of the increase in the mass of the stem. This increase is greatest at the basal end where it constitutes the callus.

In a paper previously published² the writer has called attention to the fact that when a piece of stem is left in connection with a leaf the inhibitory effect on the formation of roots in the leaf increases with the size of the piece of the stem but less rapidly than this. This is due to the fact that the increase in mass of the stem is not the same throughout the whole length of the stem but that it is greater at the base and possibly also (though to a lesser degree) in the region of a node.

Hence the increase in mass of a stem consists of the sum of two quantities, $c + l c_1$, where c expresses the mass of the callus which does not necessarily increase with the mass of the stem, and $l c_1$, where l is the length of the stem and c_1 the increase in the mass per cm. length of a stem with unit periphery. It is obvious why the inhibitory power of a stem increases generally with its length but less rapidly.

² Loeb, J., *Ann. Inst. Pasteur*, 1918, xxxii, 1.

Figs. 2 and 3 show the distribution of the reddish pigment formed in the leaves suspended in air and drying out. The reddish pigment is indicated by the stippled area in the leaf. In the two leaves connected with a piece of stem where the regeneration of shoots in the leaf is inhibited the pigment flows into the stem and the newly forming shoots in the stem. The old leaf contains pigment only in that part which is close to the petiole and this is obviously pigment in

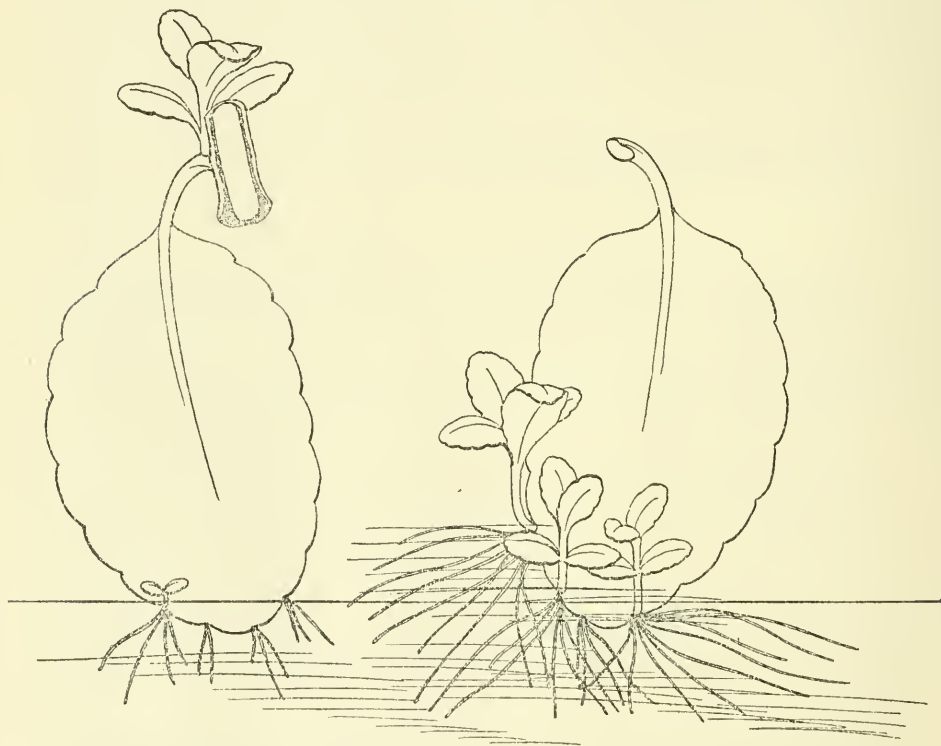


FIG. 1. Leaves suspended with apex in water. Inhibitory influence of a piece of half stem on shoot and root formation in leaf connected with it. The leaf to the right (without stem) has formed three vigorous shoots and numerous roots. The sister leaf with a small piece of split stem attached has formed in the same time a tiny shoot and a few roots. The material required for root and shoot formation in the leaf has migrated into the stem and gives rise there to an axillary shoot and to the growth in the stem indicated by stippling. Duration of experiment, Mar. 30 to Apr. 27.

the process of flowing into the stem. The two sister leaves without stems have formed shoots and roots in their notches, one at the base and the other on one side. The reddish pigment is collected near the



FIG. 2. Two sister leaves suspended entirely in air. The stippling indicates the reddish pigment. In the leaf to the left which is connected with a piece of stem the pigment flows into the axillary shoot of the stem, into the petiole, and is collected in the upper part of the leaf nearest the petiole. In the sister leaf without stem it collects near the place where the new shoots are formed and in the new shoots. Duration of experiment, Feb. 17 to Apr. 5.

newly formed shoots and on its way to these organs. These observations support the assumption that the inhibitory effect of the piece of stem on shoot formation in a leaf is due to the fact that the material available for shoot formation in the leaf flows naturally into the

stem. It also illustrates the principle that when in one part of the leaf growth is very rapid the growth in other parts of the leaf is retarded or inhibited for the reason that the flow of material is towards the rapidly growing organs.

These drawings were made later than those given in a previous paper,³ which were for this reason less striking.

The inhibitory effect of a small piece of stem on a leaf does not last permanently. It is possible that at first the piece of stem grows in the way described thus inhibiting growth in the leaf, but that later

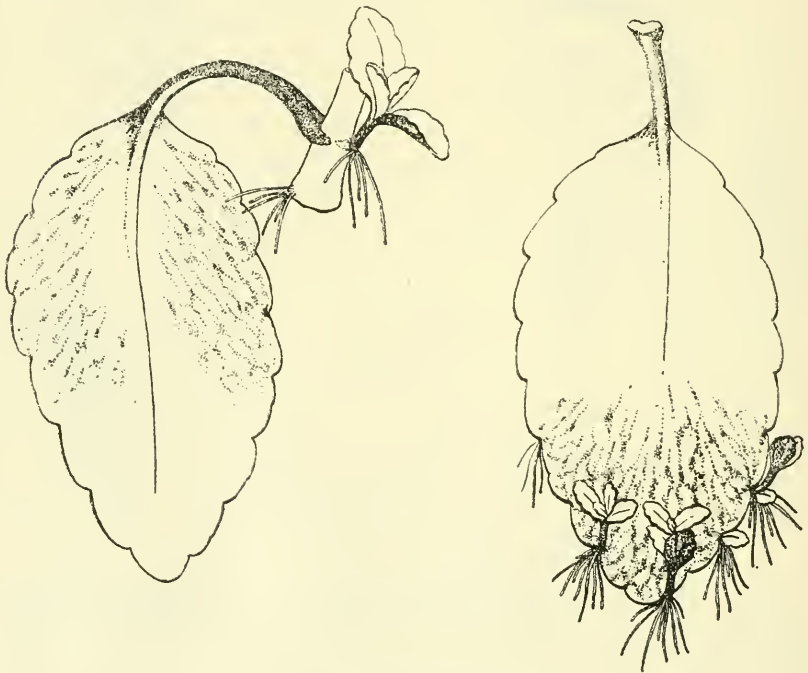


FIG. 3. The same as Fig. 2.

the rate of growth in the piece of stem diminishes, and parallel with this the inhibition of the piece of stem on the growth of roots and shoots in the leaf diminishes also.

³ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 373.

SUMMARY.

This paper contains the results of a reexamination of a law expressed in a previous paper; namely, that when a piece of stem inhibits the growth of shoots and roots in a leaf connected with it the dry weight of the stem increases and that this gain equals approximately the mass of shoots and roots the leaf would have produced if it had been detached from the stem. This has been confirmed and it has been shown that the gain of the stem as a rule even exceeds slightly the mass of shoots and roots the leaf would have produced if it had not been inhibited by the stem. This supports the idea that the inhibitory influence of the stem upon the formation of roots and shoots in the leaf is due to the fact that the material available and required for this process naturally flows into the stem.



THE REVERSAL OF THE SIGN OF THE CHARGE OF COLLODION MEMBRANES BY TRIVALENT CATIONS.

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(Received for publication, May 19, 1920.)

I.

The reversal of the sign of charge of collodion membranes (treated previously with a protein) by hydrogen ions has been discussed in a preceding paper¹ and in this paper the influence of trivalent cations on the reversal will be considered. We shall omit the discussion of the action of tetravalent cations (*e.g.* ThCl_4) since their solutions have so high a concentration of hydrogen ions that this alone suffices to bring about a reversal in the sign of charge.

We will prove first that collodion membranes which have previously been treated with a protein give the reversal in the sign of charge in the presence of trivalent cations, while collodion membranes not so treated do not show the reversal. When a solution of CaCl_2 of not too high a concentration (*e.g.* below $\text{M}/8$) is separated from pure water by a collodion membrane, which is negatively electrified, the solution shows no attraction for water, while it attracts water powerfully when the membrane is charged positively. The Ca ion acts as if it repelled positively charged water and as if it attracted negatively charged water. To find out whether or not trivalent cations reverse the sign of charge of the membrane we have to add a low concentration of a "neutral" salt with trivalent cation to the solution of CaCl_2 . Weak solutions of CeCl_3 and LaCl_3 satisfy this condition. The solutions of CaCl_2 are for this purpose made up in $\text{M}/1,024$ solutions of CeCl_3 or LaCl_3 instead of in distilled water; and the distilled water surrounding the collodion bag is also replaced by

¹ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 577.

$M/1,024$ CeCl_3 , so that in regard to CeCl_3 or LaCl_3 the liquid is the same on both sides of the membrane. Any osmotic effect can therefore only be due to the CaCl_2 . This allows us to investigate the question of the reversal of the sign of charge of the membrane by the Ce ions. Fig. 1 shows that the CeCl_3 can only reverse the sign of charge of the membrane if the latter has previously been treated with gelatin. The lower curve gives the initial rate of diffusion of water (after 20 minutes) into CaCl_2 solutions (of different concentra-

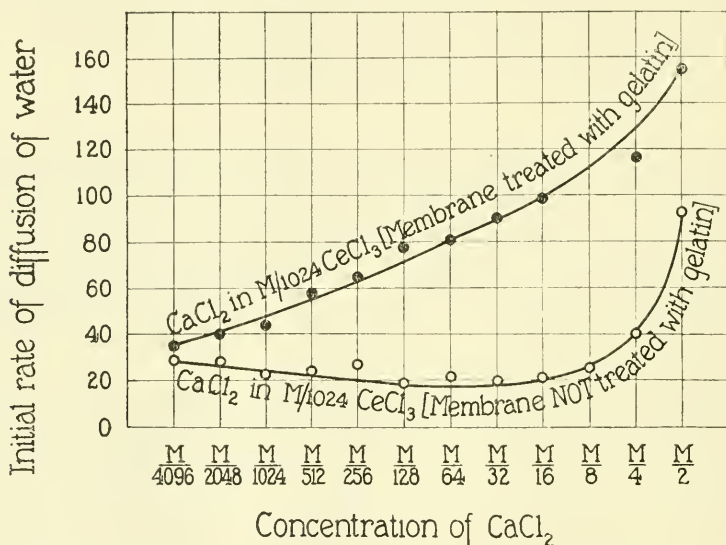


FIG. 1. Showing that CeCl_3 renders a collodion membrane positive only when it has been treated with a protein, since CaCl_2 below $M/16$ attracts water only when the membrane is positively and the water negatively charged.

tion) through collodion membranes not treated with gelatin. The membrane is negatively charged since no rise of the level of water in the solution occurs as long as the concentration of CaCl_2 is below $M/8$. The rise of the curve at a higher concentration has a different cause which need not be considered in this connection. The upper curve gives the influence of the same CaCl_2 solutions on the rate of diffusion of water from pure water into solution when the membrane has previously received a gelatin treatment. In this case the level of liquid rises in the solution and the more so the higher

the concentration of the CaCl_2 solution. The CeCl_3 has therefore caused a reversal in the sign of charge of the membrane making the latter positive. This made it possible for the CaCl_2 solution to increase the rate of diffusion of water through the membrane into the solution. The pH of the solutions varied between 5.1 (in the lowest concentration of CaCl_2) and 5.9 (for the highest concentration); *i.e.*, the solutions were all on the alkaline side of the isoelectric point of gelatin.

Experiments with electrical endosmose confirmed the conclusion that in the presence of CeCl_3 the membrane assumes a positive charge when the membrane had previously been treated with gelatin, but that the membrane remains negatively charged when it has not been treated with a protein. In these experiments with electrical endosmose the solutions inside and outside the collodion bag were solutions of CeCl_3 of identical concentration.

II.

We have shown in another paper² in this number of the *Journal* that in the case of collodion membranes treated with gelatin and rendered positive by acid the rate of diffusion of water from the side of pure water through the membrane to the solution is raised by cations in the order $\text{K} < \text{Na} < \text{Li} < \text{divalent cations} < \text{trivalent cations}$. Since the Ce ions render the gelatin film of the collodion membrane positive the influence of different cations on the attraction of water should increase in the same order as when the membrane is rendered positive by acid; and the attraction of water by the solution should be a minimum in the case of K. Fig. 2 shows that this is true. In these experiments different concentrations of the four salts, KCl, NaCl, LiCl, and CaCl_2 , from $\text{M}/2,048$ to 1M were made in $\text{M}/1,024$ CeCl_3 . The H_2O surrounding the collodion bags containing the solutions was replaced by $\text{M}/1,024$ CeCl_3 to eliminate the osmotic effect of CeCl_3 in the experiments. The experiments show that the attraction of water by the four salts follows the order we should expect if Ce caused the membrane to assume a positive charge; *i.e.*, $\text{K} < \text{Na} < \text{Li} < \text{Ca}$. The pH in these experiments varied from

² Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 673.

5.2 to 6.0, thereby proving that the Ce reversed the sign of the charge of the gelatin film on the inside of the membrane without raising the hydrogen ion concentration to that point where an acid reversal occurs. In Fig. 3 M/1,024 AlCl_3 solutions are used for rendering the membrane positive. In this case the pH was about 4.1 in all solutions.

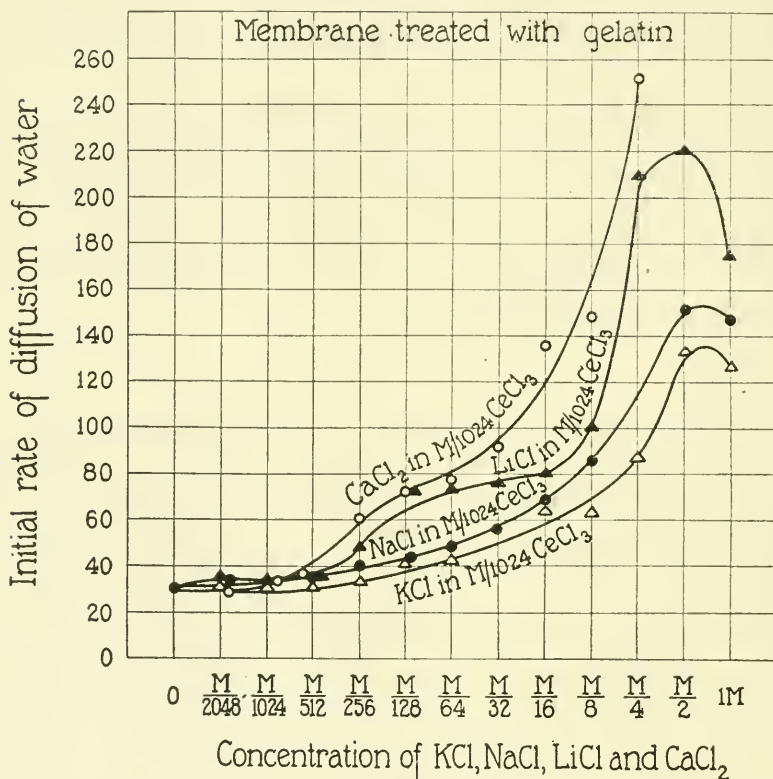


FIG. 2. The relative influence of different cations upon the attraction of water is in the order $\text{Ca} > \text{Li} > \text{Na} > \text{K}$, proving that the membrane is positively charged in the presence of Ce.

The order of efficiency of cations remains the same as when CeCl_3 is used, but the effect is quantitatively larger in Fig. 3 than in Fig. 2.

All these experiments and many others show that trivalent cations cause the membrane previously treated with gelatin to be charged positively on the alkaline side of the isoelectric point of gelatin.

III.

The next question was as to the minimal concentration of a trivalent cation required to render the membrane positive. For this purpose

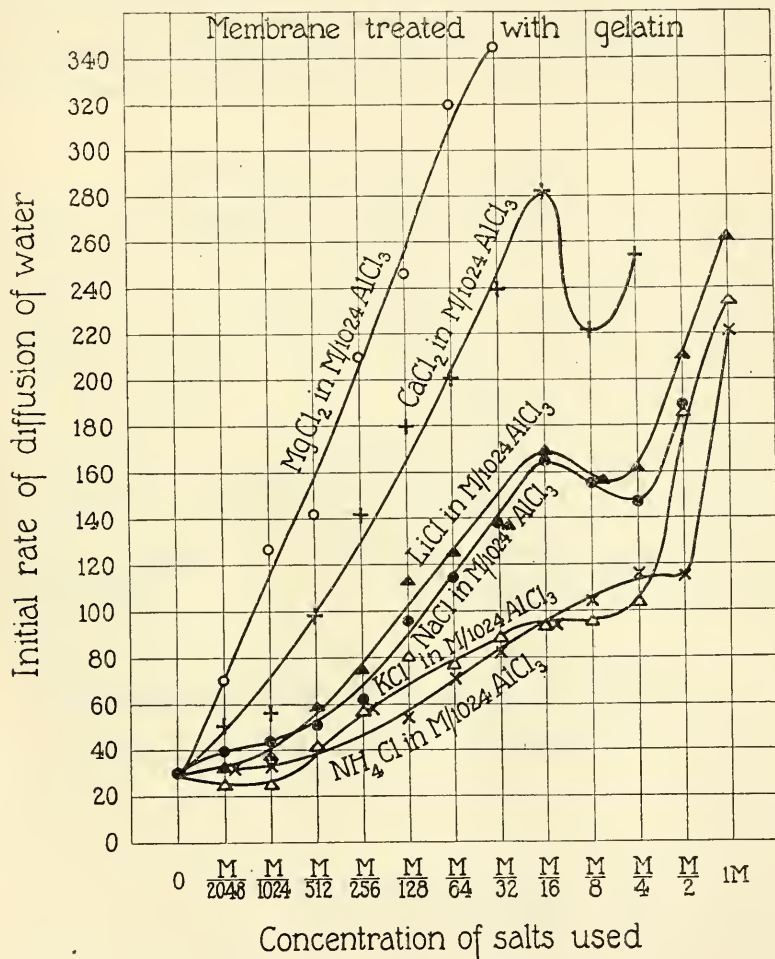


FIG. 3. Showing that Al charges the membrane positively.

various concentrations of CeCl_3 were prepared, beginning with $\text{M}/65,536 \text{ CeCl}_3$. To each of these solutions so much CaCl_2 was added that the concentration was $\text{M}/256$ in regard to CaCl_2 . These

solutions were put into collodion bags previously treated with gelatin. The bags were put into beakers containing the same solutions of CeCl_3 as that inside the bag but free from CaCl_2 . Hence the Ce acted only on the sign of the charge of the membrane but not on the attraction of water. This latter was done by the CaCl_2 . Whenever the level of the solution of CaCl_2 rises we know the membrane must be positively charged. The curve in Fig. 4 shows that the

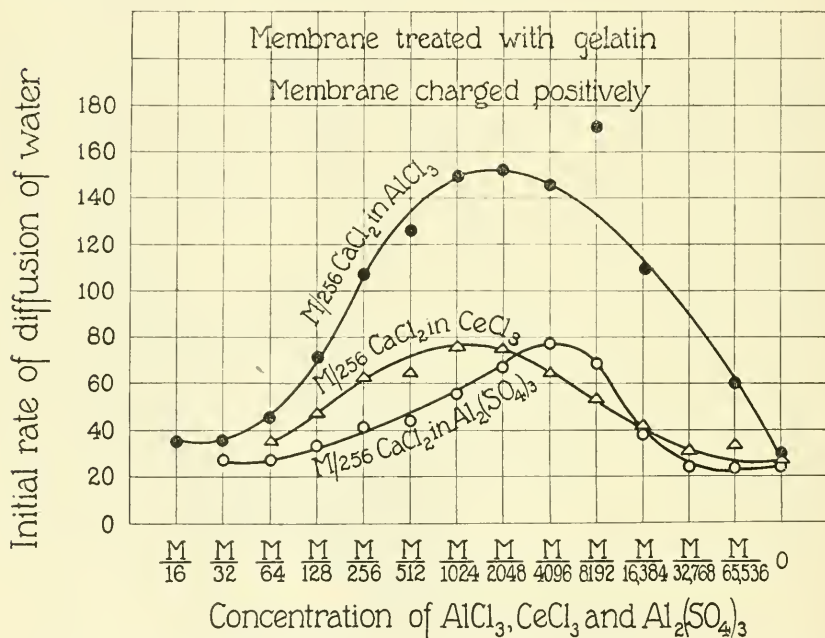


FIG. 4. Lowest concentration at which Ce and Al charge the membrane positively.

membrane already assumes a positive charge in $M/32,768 \text{ CeCl}_3$ and probably in lower concentrations of CeCl_3 .

When the CeCl_3 was replaced by AlCl_3 the membrane assumed a positive charge when the concentration of AlCl_3 was less than $M/65,536$. The pH of this latter solution of AlCl_3 was 5.0, so that the reversal was due to the Al ion and not to the hydrogen ion concentration. The fact that Al is more efficient than the Ce (or La) ion may be connected with the fact that the ionic radius of Al is considerably

smaller than that of either Ce or La. The drop in the curves when the concentration of the CeCl_3 or AlCl_3 solution exceeds $M/1,024$ is due to the concentration of the anion, as is shown by the fact that the drop is greater in the case of $\text{Al}_2(\text{SO}_4)_3$ than in the case of AlCl_3 . This latter phenomenon has been discussed in previous papers. The pH of the $\text{Al}_2(\text{SO}_4)_3$ solutions almost coincided with that of the AlCl_3 solutions of the same concentration.

IV.

It is therefore certain that the trivalent ions bring about a positive electrification in the collodion membrane in contact with water when the membrane has been treated with a protein, but that they cannot reverse the sign of the charge of collodion membranes not so treated. This indicates that the reversal is at least partly due to an action of the trivalent cations on the protein. A chemical combination can only occur between Ce or Al and gelatin on the alkaline side of the isoelectric point where the gelatin is capable of combining with metals. In the experiments described thus far the pH of the CeCl_3 solutions was with one exception always > 4.7 . The question arises, how will a CeCl_3 solution act on the acid side of the isoelectric point of the protein forming the inner lining of the collodion bag where the membrane is already rendered positive by the acid?

$M/256$ LaCl_3 solutions were prepared at different pH, from 7.0 to 2.6, by adding KOH or HNO_3 to the distilled water used for the solutions as required for the pH. These solutions were put into collodion bags and the latter were dipped into H_2O of exactly the same pH as that of the solutions inside the bag. The initial rise of water (in the first 20 minutes) inside the bags was then observed. Similar experiments were made with CeCl_3 . The collodion membranes had previously been treated with gelatin. Fig. 5 gives the results. It is plain that the curves consist of two distinct parts and that the dividing line seems to lie near the isoelectric point of gelatin. On the alkaline side of the point, *i.e.* for pH 4.7 or above, the initial rate of diffusion is near 140 mm.; and it varies very little with a change of pH. As soon, however, as the pH falls to 4.7 or below, the rate of diffusion rises steeply to 300 and 340 mm. for the two salts.

To understand this result we must discriminate between the effects of the trivalent cations on the reversal of the sign of charge and on the increase of the rate of diffusion of water into the solution, which seems to be due to an increase in the density of charge of a membrane already positive. On the alkaline side of the isoelectric

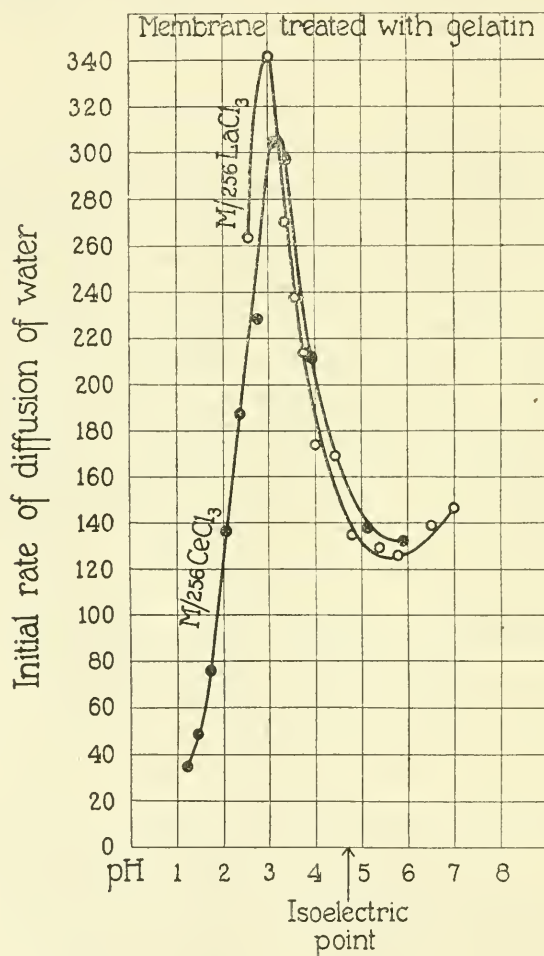


FIG. 5. Although $M/256$ solutions of La and Ce charge the membrane positively on the alkaline side of the isoelectric point of gelatin, their attraction for water rises very steeply on the acid side of the isoelectric point of gelatin.

point of gelatin the trivalent cation makes the membrane positive by combining with gelatin and forming a salt; *e.g.*, La gelatinate. But, in addition, the salts of trivalent cations attract the water, and the more the higher their concentration until a certain maximum is reached, as shown in Fig. 4. On the acid side from the isoelectric point the protein film cannot react chemically with the La or Ce and the membrane is charged positively through the influence of the

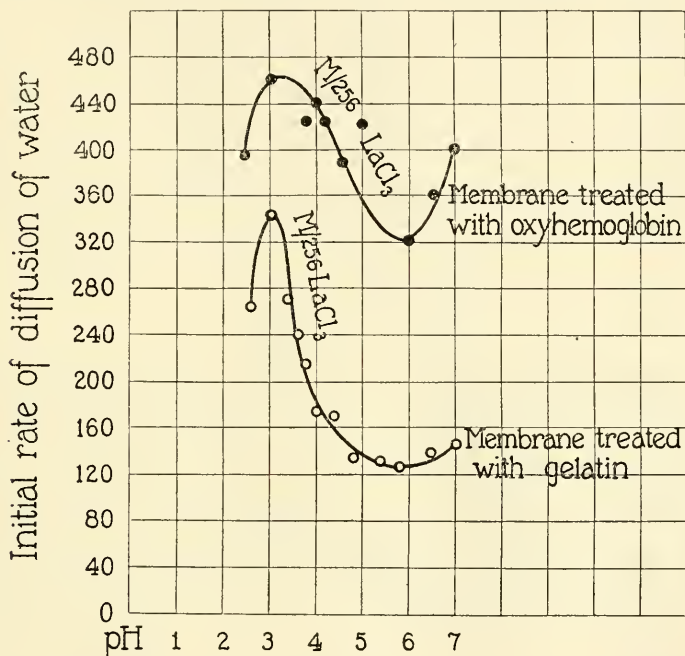


FIG. 6. Showing that the critical point for this steep rise varies with the isoelectric point of the protein with which the membrane has been treated.

acid. In this case the trivalent cation acts apparently by increasing the density of charge of the positive membrane considerably, as was to be expected.

In order to test this idea further, membranes treated with oxyhemoglobin instead of with gelatin were tried. The isoelectric point of oxyhemoglobin is at $\text{pH} = 6.8$. In this case the steep rise in the curve with LaCl_3 should be nearer this value than 4.7. The curves

of Fig. 6 show that this is the case. It may be mentioned incidentally that the initial rate of diffusion of water into solutions was greater when the membranes had been treated with oxyhemoglobin than when they had been treated with gelatin. We shall return to this phenomenon in another connection.

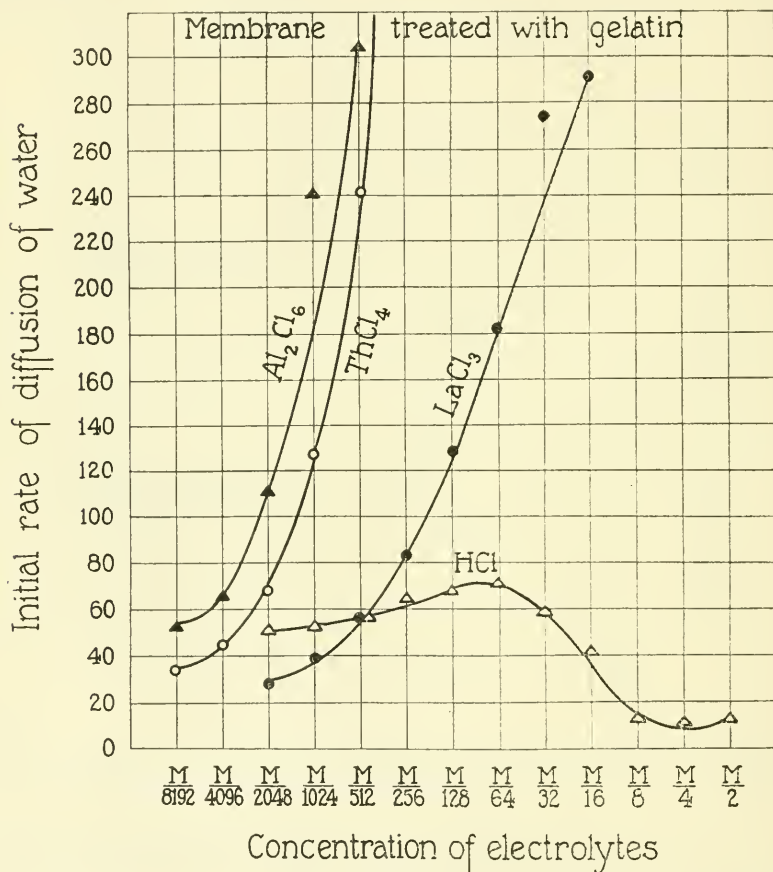


FIG. 7. Showing that the attraction of salts with trivalent cations for water is considerably greater than the attraction of acid (HCl) for water, although acids are very efficient in making the membrane positive.

V.

The experiments represented in Figs. 5 and 6 seem to show that the efficiency of ions in causing the reversal of the sign of charge is as great in the case of hydrogen ions as in the case of trivalent cations; while the influence on the rate of diffusion of water is considerably greater in the case of trivalent cations than in the case of hydrogen ions. Fig. 7 permits a comparison of the attraction of water by solutions of equal concentration of HCl , LaCl_3 , Al_2Cl_6 , and ThCl_4 through membranes treated with gelatin. On one side of the membrane was the solution, on the other pure water. If we consider only concentrations up to $M/32$ this attraction is very slight in the case of HCl as compared with LaCl_3 or Al_2Cl_6 . (It should be stated that in the experiments with HCl the level of the water was considerably higher at the beginning of the experiment than in the other solutions.)

Theoretical Remarks.

The experiments have shown that a reversal of the sign of charge of a collodion membrane can be brought about by acids and by "neutral" salts with trivalent cation after the inside of the membrane has been in contact for an hour or more with a sufficiently strong solution of a protein whereby apparently a protein film is formed on the inside of the membrane. Collodion membranes not treated are always negatively charged in contact with water, no matter whether or not hydrogen ions and trivalent cations are present in sufficient concentration. Collodion membranes when treated with a protein are also negatively charged when the reaction of the solution is on the alkaline side of the isoelectric point of the protein and when the solution is free from trivalent or tetravalent cations. In this case the protein exists in the form of a metal proteinate dissociating into a positive metal ion, the protein adhering to the inside of the collodion membrane. This might suggest that the membrane becomes negative in contact with water on account of these metal ions dissolving in water, while the solid film of protein anions adhering to the membrane is negatively charged. When the hydrogen ion concentration is raised sufficiently to cause the transformation of the protein

film into a protein-acid salt the reversal in the sign of charge of the membrane would be easily intelligible since the protein-acid salt dissociates into a positive protein cation forming the surface film of the membrane, and an anion which goes into solution.

A difficulty arises, however, if we try to explain the reversal of the sign of charge of the membrane by trivalent cations on the alkaline side of the isoelectric point of the protein. In this case the protein film consists of La or Ce proteinates which are practically insoluble. Practically no dissociation into a negative protein anion and a trivalent cation would be supposed to occur and it seems not possible to state why an insoluble La proteinate should assume a positive charge when in contact with water. As long as this fact is not explained it remains doubtful whether the tentative explanation just given for the acid reversal is correct or complete. It also remains to be explained why the collodion membrane not treated with a protein always assumes a positive charge regardless of the hydrogen ion concentration or the concentration of trivalent cations. It might be argued that the collodion membrane differs from proteins in not being an amphoteric electrolyte and in not being able to combine with trivalent cations. Another possibility must, however, be considered; namely, that the hydrogen ions and trivalent cations influence the surface electrical potential of the solution and that the combined effects of these ions on the surface electrical potential of the membrane and of the liquid determine the phenomena described in our papers.

SUMMARY.

1. Trivalent cations cause a collodion membrane covered with a protein film to be charged positively while they do not produce such an effect on collodion membranes not possessing a protein film. The same had been found for the reversal of the sign of charge of the membrane by acid.

2. This reversal in the sign of charge of the membrane by trivalent cations occurs on the alkaline side of the isoelectric point of the protein used; while the reversal by acid occurs on the acid side of the isoelectric point.

3. The reversal seems to be due to or to be accompanied in both cases by a chemical change in the protein. The chemical change which occurs when the hydrogen ions reverse the sign of charge of the protein film consists in the formation of a protein-acid salt whereby the H ion becomes part of a complex protein cation; while the chemical change which occurs when trivalent cations reverse the sign of charge of the protein film consists in the formation of an insoluble and therefore sparingly or non-ionizable metal proteinate.

IONIC RADIUS AND IONIC EFFICIENCY.

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(Received for publication, May 4, 1920.)

I. INTRODUCTION.

When pure water is separated from solutions of electrolytes of not too high a concentration (generally below $M/4$) by a collodion membrane, the initial rate of diffusion of water through the membrane from the side of pure water to that of the solution is influenced in a characteristic way by ions and the following rules have been found to govern this influence.

1. Ions possessing the same sign of electrical charge as the membrane increase and ions with the opposite sign of charge diminish the initial rate of diffusion of water.

2. The relative influence of the oppositely charged ions is not the same for all concentrations. In the lowest concentrations of electrolytes the influence of the ions with the same sign of charge prevails, increasing at first with increasing concentration of the electrolyte until a maximum is reached; a further increase in concentration diminishes the rate of diffusion of water into the solution and the more so the higher the concentration of electrolyte. The turning point varies for different electrolytes but seems to lie for a number of neutral solutions at about $M/256$ or above.

3. The influence of ions on the initial rate of diffusion of water from the side of pure water through the membrane into the solution increases with their valency and with a second constitutional quantity which in the earlier papers I designated arbitrarily as the radius of the ions.

It will be shown in this paper that the second constitutional quantity seems indeed to be, for monatomic and monovalent ions, the radius of the ion and that the rule connecting the efficiency of ions with

their radius is as follows: *The efficiency of anions increases directly and that of cations inversely with the radius of the ion.* By radius of the ion we mean the distance between the positive nucleus of an ion and its outermost ring or shell of electrons. This term has a definite meaning in the case of monatomic ions.

The method of experimentation is the same as in the preceding publications. The solution of the electrolyte was put into collodion flasks of about 50 cc. content, which had previously been filled over night with a 1 per cent gelatin solution. The gelatin solution was then carefully washed out with warm water. The membrane retained on its inside a very thin film (probably of only one molecule in thickness) of gelatin. This was done to enable us to reverse the sign of charge of the membrane with dilute acid; when the hydrogen ion concentration is below $2 \times 10^{-5} N$ the membrane is negatively (and the watery phase positively) charged; while when the hydrogen ion concentration is above this value the membrane assumes a positive and the water a negative charge. The membranes used in the experiments to be described had therefore all been treated with gelatin.

The collodion flask was closed with a rubber stopper, through which a glass tube with a bore of about 2 mm. in diameter was pushed inside the flask. The latter was filled with the solution of electrolyte and suspended in a beaker with distilled water having the same hydrogen ion concentration as the solution of electrolyte. The glass tube served as a manometer to indicate the rate at which water diffused from pure water into the solution through the collodion membrane. The temperature was kept constant at 24°C. We shall treat the influence of ions on negatively and positively charged membranes separately.

II. Negatively Charged Membranes.

When an electrical double layer is formed at the boundary of a collodion membrane (previously treated with gelatin as described) the membrane assumes a negative and the watery phase a positive charge as long as the hydrogen ion concentration is below $2 \times 10^{-5} N$ and no trivalent or tetravalent cations are present. If the hydrogen ion concentration exceeds $2 \times 10^{-5} N$ and becomes $10^{-4} N$ the sign of charge on the two layers is reversed. This was proved by experi-

ments on electrical endosmose.¹ We will first describe experiments in which the membrane is negatively and the watery phase positively charged.

We have shown in a previous paper that anions accelerate and cations depress the rate of diffusion of water through negatively charged membranes.² Fig. 1 shows the relative influence of the anions of three potassium salts, KCl, KBr, and KI on the rate of diffusion of water through collodion membranes. The ordinates

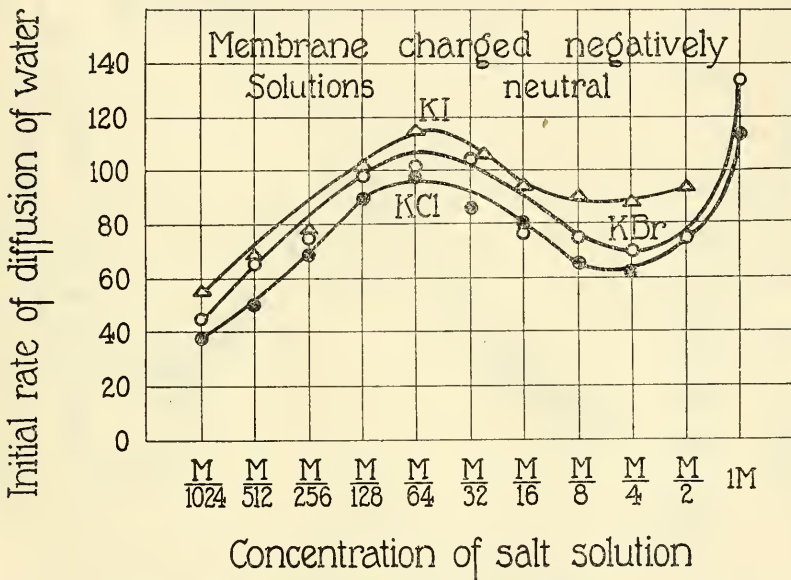


FIG. 1. The rate of diffusion of water through a negatively charged membrane (from the side of pure water to solution) and the density of the charge of the latter are increased by monovalent, monatomic anions in the order $I > Br > Cl$. Solutions almost neutral (pH about 6.0).

indicate the rise of liquid in the manometer in 20 minutes; the abscissæ indicate the concentration of the solution. The salt solutions as well as the pure water separated from the solution by the collodion membrane were almost neutral, having a hydrogen ion concentration of about 10^{-6} N. It is obvious that the accelerating influence of

¹ Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 577.

² Loeb, J., *J. Gen. Physiol.*, 1919-20, ii, 173.

anions on the rate of diffusion of water from pure water into the solution through a negatively charged membrane increases in the order $I > Br > Cl$, the accelerating influence of the I ion being the greatest.

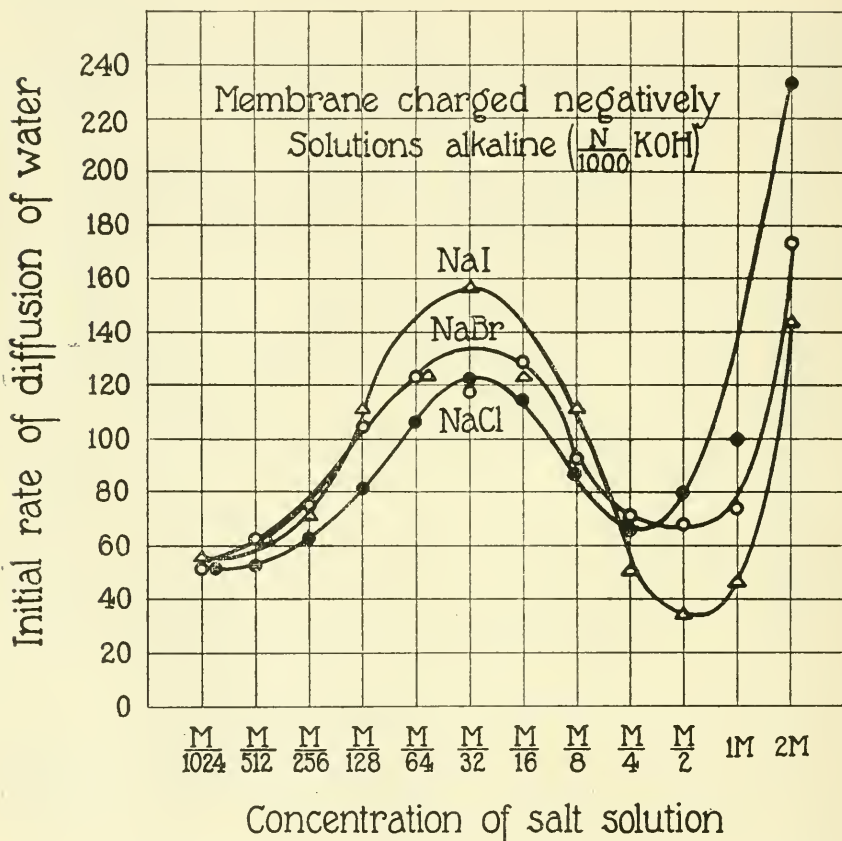


FIG. 2. The same as Fig. 1 except that the solutions and water are alkaline (pH about 11.0).

The same is true when the solution is more alkaline. Thus in Fig. 2 NaCl, NaBr, and NaI were dissolved in a $N/1,000$ solution of KOH instead of in pure water and the outside solution was $N/1,000$ KOH. The sign of charge of the membrane remained negative, however, and therefore the relative efficiency of the three anions

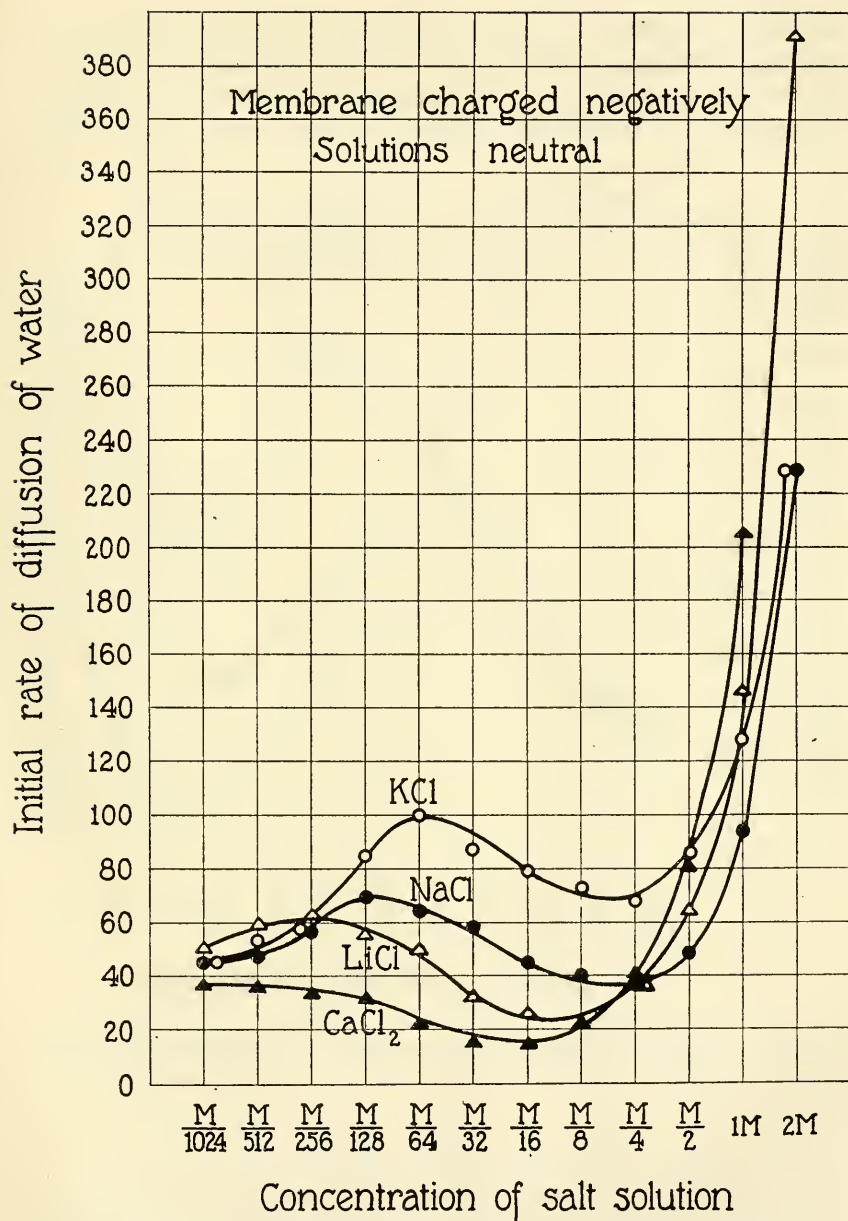


FIG. 3. The rate of diffusion of water through a negatively charged membrane and the density of charge of the latter are depressed by monovalent, mono-atomic cations in the order $\text{Li} > \text{Na} > \text{K}$. Solutions neutral (pH about 6.0).

remained the same. The rate of diffusion of water was greater in the case of solutions of NaI than in the case of NaBr, and greater in NaBr than in NaCl. Hence the statement is confirmed that when a membrane is negatively charged the rate of diffusion of water

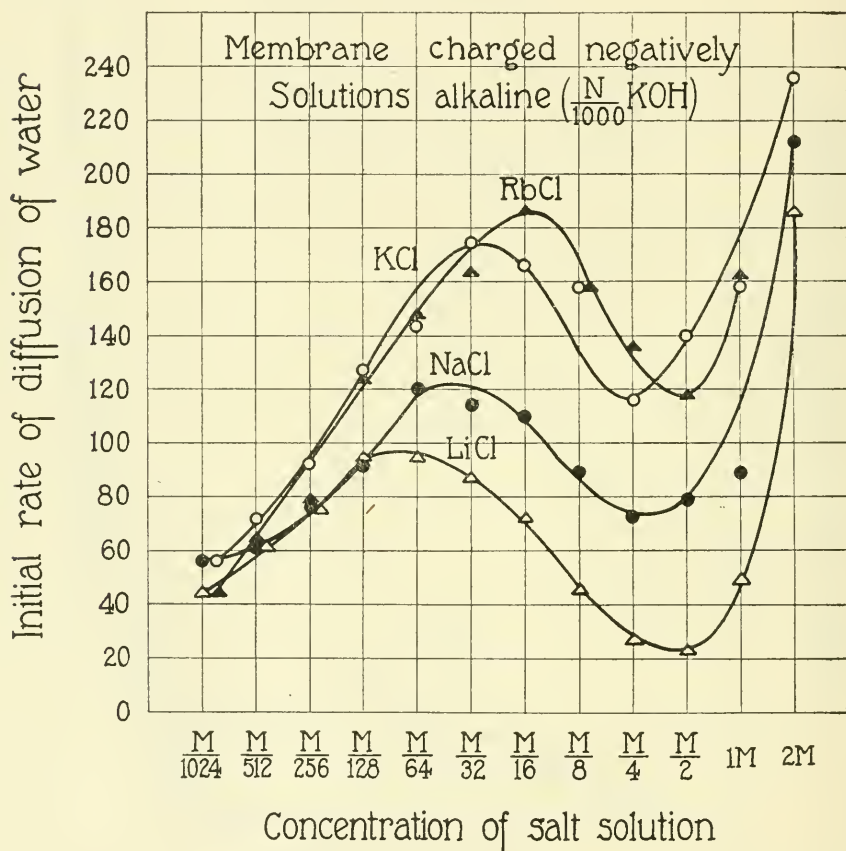


FIG. 4. The same as in Fig. 3 when solutions are alkaline (pH about 11.0). Order of depressing action of cations on negatively charged membrane $\text{Li} > \text{Na} > \text{K} > \text{Rb}$.

through the membrane from pure water into solution increases for the anions I, Br, and Cl in the order $\text{I} > \text{Br} > \text{Cl}$.

Since this is also the order of the magnitude of the ionic radius of the three anions, I having the greatest radius, we can say that the

accelerating effect of monatomic, monovalent anions upon the rate of diffusion of positively charged water through negatively charged membranes from the side of pure water to the side of the salt solution increases directly with the order of magnitude of the radius of the anion.

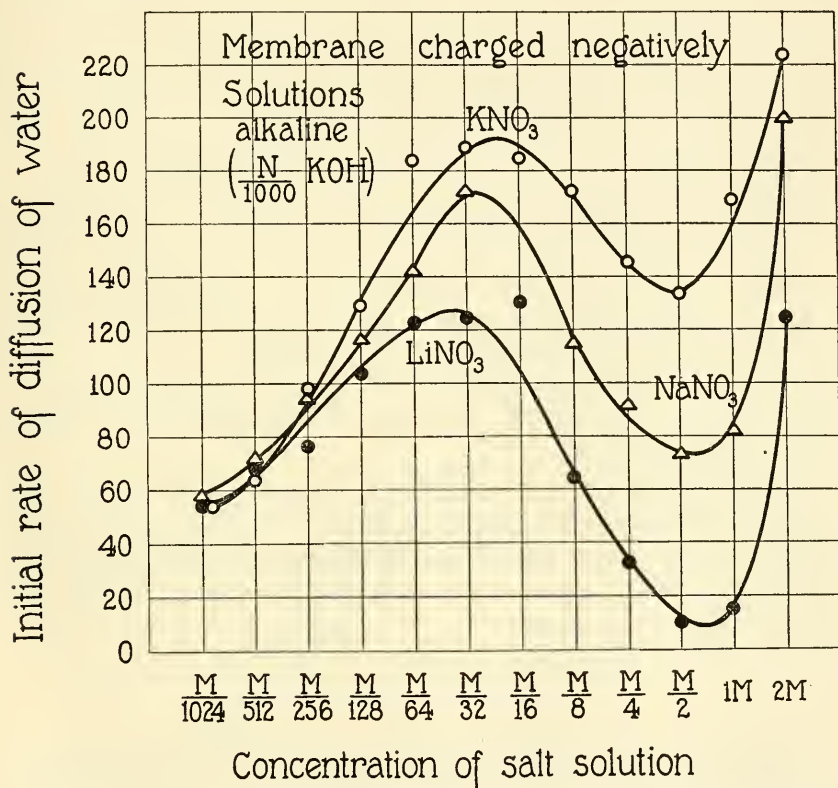


FIG. 5. The depressing order of cations upon negatively charged membranes is the same for NO_3 as for Cl (Fig. 4). Solutions alkaline, order of depressing effect $Li > Na > K$.

The same relative order of efficiency for the three anions was found also when they were in combination with Li .

While the anions increase the rate of diffusion of positively charged water through a negatively charged membrane cations diminish it and the question arises in what order the depressing effect of the

monovalent cations increases. Fig. 3 shows that in the case of monovalent cations the depressing effect is least in the case of K, and increases in the following order, $K < Na < Li$.

In these experiments the solutions were almost neutral (hydrogen ion concentration about $10^{-6} N$). The order of efficiency of the cations remains the same when the solutions of these salts are made up in $N/1,000$ KOH and when the distilled water in the outside solution is replaced by $N/1,000$ KOH; since in this case the membrane is also negatively and the water positively charged. Fig. 4 shows that the depressing effect of the monovalent, monatomic cations increases in the order $Rb < K < Na < Li$, where the depressing effect is least in the case of Rb, and greatest in the case of Li. This order of the depressing influence of cations is independent of the anion used as long as the anion is always the same for the different cations used. Thus Fig. 5 shows the same order of the depressing action of cations for nitrates as in Fig. 4 in the case of the chlorides.

Since this is also the order in which the ionic radius of the four ions diminishes (Rb having the greatest ionic radius) we can make the following statement.

The depressing effect of monatomic, monovalent cations on the rate of diffusion of positively charged water through a negatively charged collodion membrane from the side of pure water to the side of the salt solution increases inversely with the order of magnitude of the radius of the cation.

III. Positively Charged Membranes.

It has been shown in preceding publications that when the membrane is positively and the water negatively charged the cations increase and the anions diminish the rate of diffusion of water from the side of pure water through the membrane into the solution. In order to make a membrane, previously treated with gelatin, positively and the watery phase of the double layer negatively charged it is necessary to give the water and the solutions a hydrogen ion concentration of about $10^{-4} N$ or above. We made the solution acid by dissolving the salts in $10^{-3} N$ HNO_3 and the distilled water outside the collodion bag was also replaced by $10^{-3} N$ HNO_3 . The hydrogen

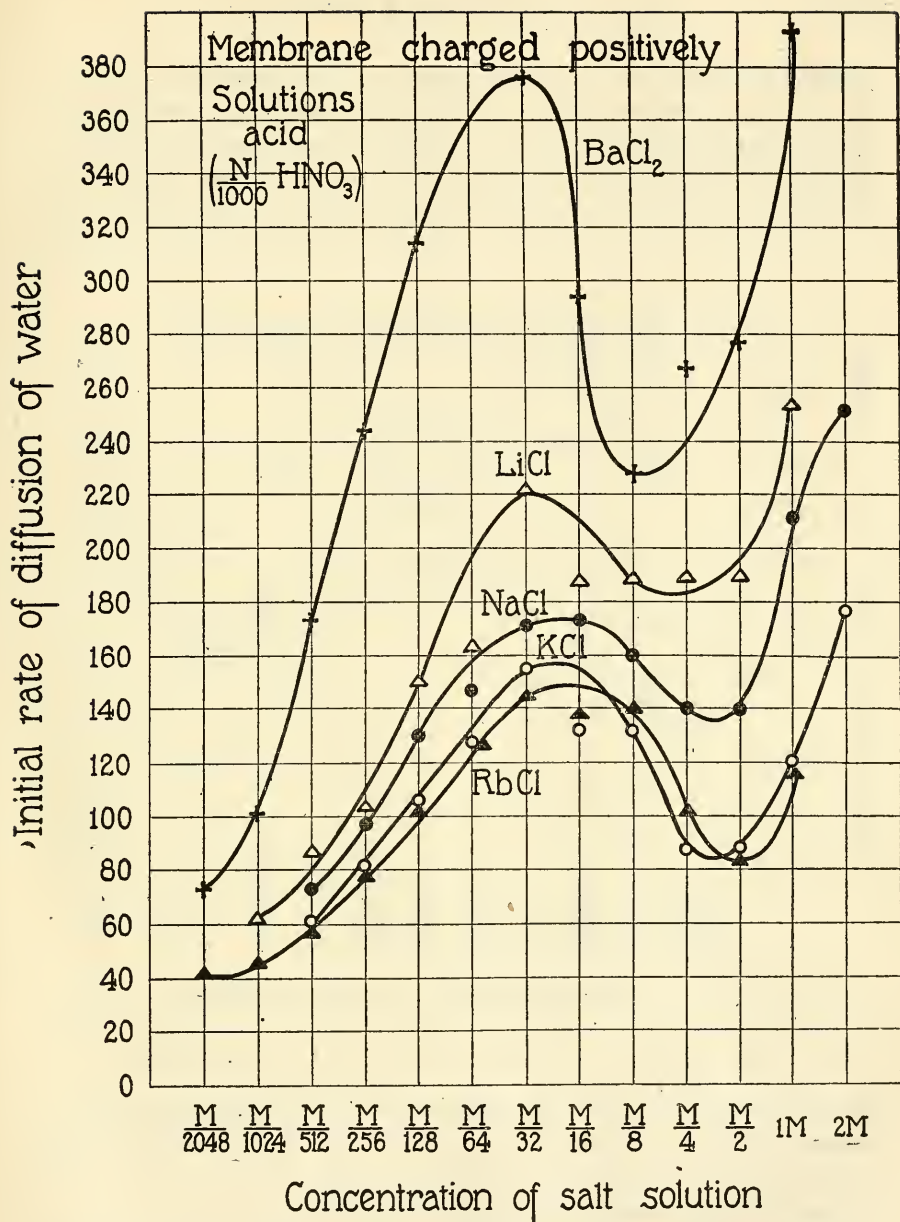


FIG. 6. The rate of diffusion of water through a positively charged membrane and the density of charge of the membrane are increased by the monovalent, monatomic cations in the order $\text{Li} > \text{Na} > \text{K} > \text{Rb}$. Solutions acid (pH about 3.0).

ion concentration of the solution inside the collodion flask and the water surrounding the collodion flask had therefore the same hydrogen ion concentration of about 10^{-3} N. Fig. 6 shows the order of influence of different cations upon the rate of diffusion of water through the membrane into the solution. The anion is always the same; namely, Cl. The order of efficiency of the cation series increases in the order

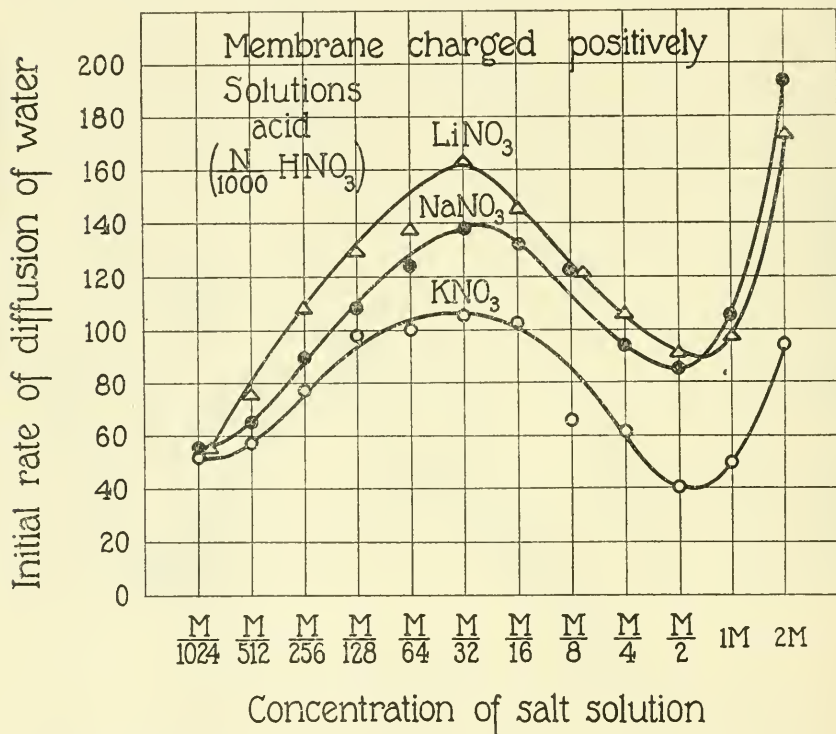


FIG. 7. The same order of efficiency of cations if the anion is NO₃.

Rb < K < Na < Li; *i.e.*, the efficiency of monovalent, monatomic cations increases inversely with the magnitude of their radius. This order is the same regardless of the anion. Thus Fig. 7 shows that the order is the same when Cl is replaced by NO₃.

The anion depresses the rate of diffusion of negatively charged water through the membrane into the solution when the membrane is positively charged. Fig. 8 shows that this depressing effect of

different potassium salts increases in the order $I > Br > Cl$, where the depressing effect of I is greatest.

This order is the same regardless of the nature of the cation. Thus Fig. 9 shows the same order of depressing effect of the anion on the rate of diffusion of water through positively charged membranes in the case of barium salts.

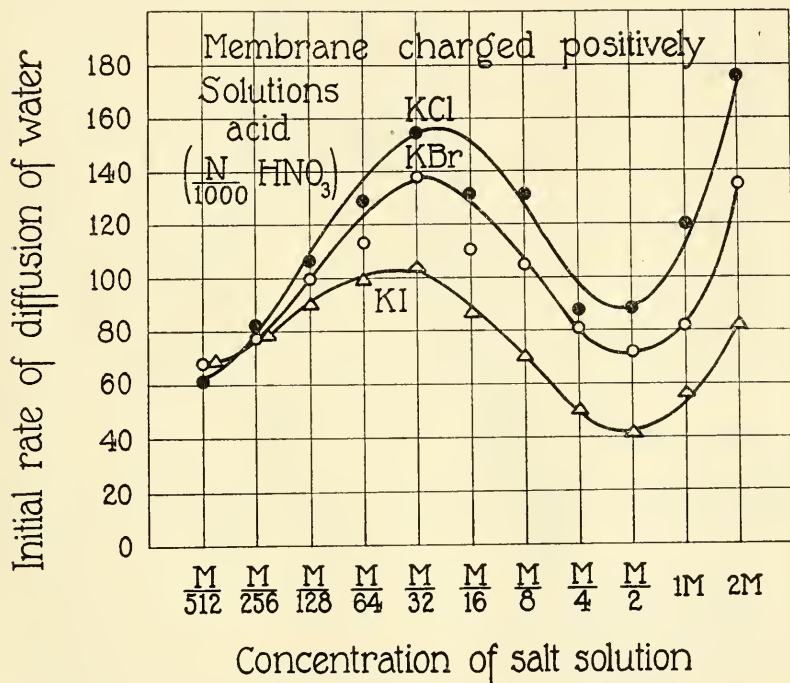


FIG. 8. The rate of diffusion of water through positively charged membranes (pH about 3.0) and the density of charge of membrane are depressed by anions in the order $I > Br > Cl$.

If we combine the results of the experiments on positively and negatively charged membranes we can express them in the following form.

The accelerating and depressing effect of monatomic, monovalent ions on the rate of diffusion of water through a collodion membrane from the side of pure water to the side of the solution increases for anions directly and for cations inversely with the magnitude of their radius.

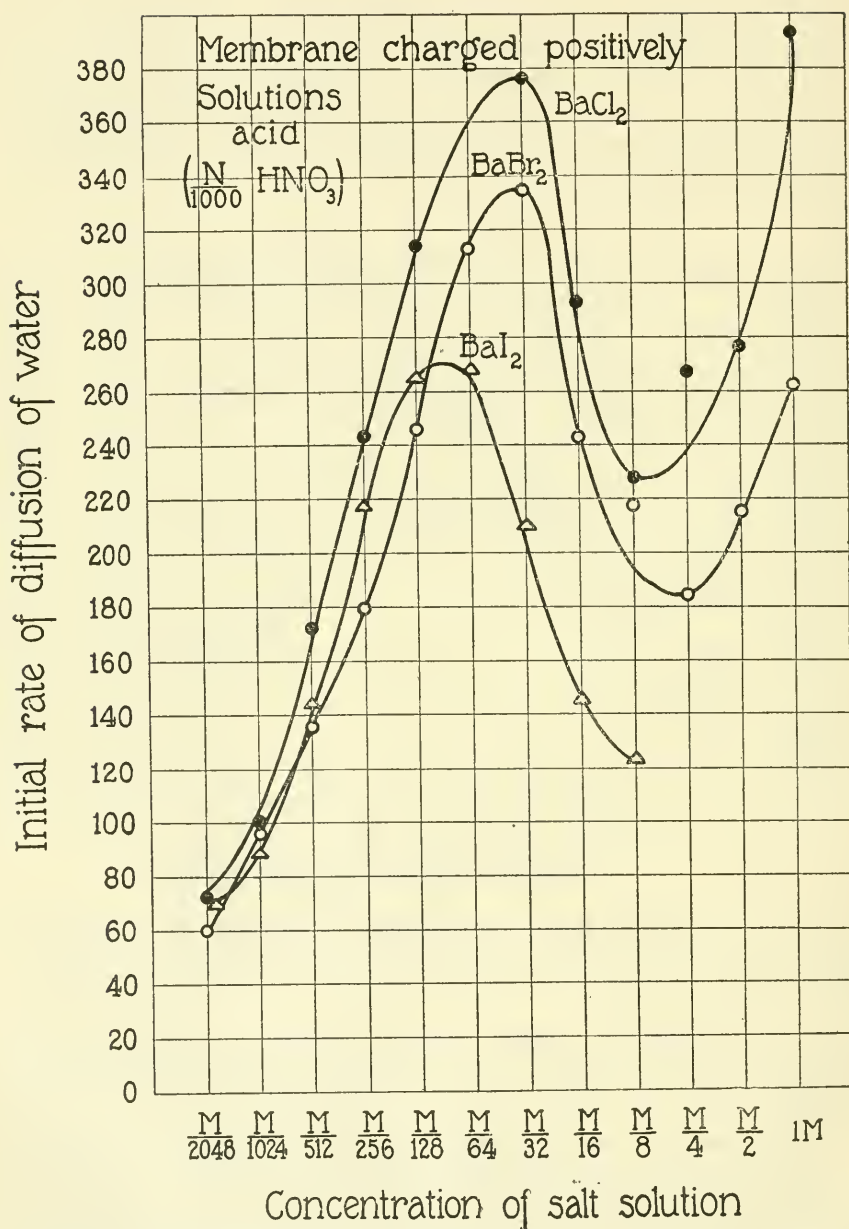


FIG. 9. The same as Fig. 8 for a different cation.

IV. Theoretical Considerations.

Kossel³ has suggested that for chemical reactions of atoms we may replace the atoms by simplified models consisting only of the charges of the positive nuclei in the center and the ring of valency electrons. The distance between the positive nucleus and the outermost ring or shell of electrons we call the radius of the atom or of the ion. While a monatomic, monovalent cation has one excess positive charge in its nucleus a monatomic, monovalent anion has one excess negative charge in its periphery. Although these positive and negative charges are alike, the relative electrostatic action of ions with the same charge must vary with the radius. Cations act through the excess positive charge on the nucleus, and the electrostatic action of the nucleus must become the greater the smaller its radius; *i.e.*, the nearer the positive nucleus can get to the body on which it is supposed to act. This explains why the accelerating as well as the depressing efficiency of a cation on the rate of diffusion of water through the membrane increases *inversely* with the radius of the cation; namely, in the order $\text{Rb} < \text{K} < \text{Na} < \text{Li}$, where Rb has the minimal effect.

When the nucleus has more than one excess charge the accelerating as well as the depressing efficiency increases with the valency, since *ceteris paribus* the electrostatic action of a cation must increase with the number of excess charges of its nucleus.

We understand also why the accelerating as well as the depressing efficiency of an anion increases *directly* with its radius, since the electrostatic effect of a monovalent anion on an outside body is determined by its excess electron and hence will be the greater the greater the distance between the valency electron and its positive nucleus.

It is also obvious why the efficiency of an anion should increase with its valency; *i.e.*, with the number of valency electrons it contains in excess of its nuclear charges.

We can only speak of ionic radius in the case of monatomic ions like K or Cl; when, however, an ion consists of more than one atom, *e.g.* NO_3 , CNS , we have more than one positive nucleus and each nucleus has its own rings or shells of electrons. The term ionic

³ Kossel, W., *Ann. Physik*, 1916, xlix, 229.

radius loses its meaning in this case unless we substitute for such polyatomic ions a monatomic model with one nucleus, one ring of valency electrons and a radius calculated in such a way as to render the electrostatic effect of the monatomic model equal to that of the polyatomic ion which it is supposed to represent.

The rule at which we arrived, namely that the efficiency of the anions increases directly with their ionic radius while the efficiency of the cations increases inversely with their ionic radius, is probably of general applicability in physical chemistry as well as in physiology, wherever the efficiency of ions depends on their electrostatic action.

There are numerous observations in the physiological literature which indicate an inversion of the order of efficiency of monovalent cations when the reaction of the solution changes from basic to acid. It is possible that these facts will find their explanation on the basis of our rule; namely, that on one side of the neutral (or in certain cases the isoelectric) point the effect observed is increased by the cation and inhibited by the anion, while on the other side it is increased by the anion and inhibited by the cation. Unfortunately the facts given are often too incomplete to test this idea and the experiments are generally done with such high concentrations of electrolytes that it is doubtful whether they can be used for any conclusions concerning the specific effects of ions.

SUMMARY.

1. It has been shown in preceding papers that when we separate solutions of electrolytes from pure water by collodion membranes the ions with the same sign of charge as the membrane increase while the ions with the opposite sign of charge diminish the rate of diffusion of water from the side of pure water to the side of solution; and that the accelerating and depressing effects of these ions on the rate of diffusion of water increase with their valency.

2. It is shown in this paper that aside from the valency a second quantity of the ion plays a rôle in this effect, namely the radius, which in a monatomic ion means the distance between the central positive nucleus and the outermost ring or shell of electrons of the ion. In monatomic, monovalent anions the radius increases in the

order $\text{Cl} < \text{Br} < \text{I}$ (being largest in I), while for monatomic, monovalent cations it increases in the order $\text{Li} < \text{Na} < \text{K} < \text{Rb}$ (being largest in Rb).

3. It is shown that the accelerating as well as the depressing effect of the anions mentioned increases directly with the order of magnitude of their radius and that the efficiency is greatest in the case of I which has the largest radius; while the accelerating as well as the depressing effect of cations increases inversely with the order of magnitude of their radius, Li with the smallest radius having the greatest efficiency.

4. This is intelligible on the assumption that the action of the ions is electrostatic in character, in the case of cations due to the electrostatic effect of the excess charge of their positive nucleus, and in the case of anions due to the excess charge of their captured electron. The electrostatic effect of the positive nucleus of a cation on the membrane (or any other body) will be the greater the smaller the ionic radius of the cation; and the electrostatic effect of an excess electron will be the greater the further its distance from its own positive nucleus.

5. It is suggested that this rule may possibly include polyatomic, monovalent ions (*e.g.* NO_3 , CNS , etc.) when we replace these polyatomic ions by monatomic models in which the radius is calculated in such a way as to give the model the same electrostatic effect which the polyatomic ion possesses.

6. These conclusions are in harmony with the fact that the efficiency of ions increases also with their valency.

7. It is suggested that these rules concerning the influence of the ionic radius can possibly be demonstrated in other phenomena, depending on the electrostatic effect of ions.

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